Volume 92 MARCH-APRIL 1953 Number 2

The Journal of Infectious Diseases

Founded 1904 by the John Rockefeller McCormick Memorial Institute for Infectious Diseases
under the Editorship of LUDVIG HEKTOEN and EDWIN O. JORDAN

WILLIAM H. TALIAFERRO, *Editor-in-Chief*

Advisory Editors

WILLIAM BURROWS · G. M. DACK · F. B. GORDON · R. W. HARRISON · STEWART R. KOSER
ROBERT M. LEWERT · CLAYTON O. LOOSLI · KARL F. MEYER · C. PHILLIP MILLER
JAMES W. MOULDER · F. G. NOVY · ROBERT RUSTIGIAN

PUBLISHED FOR THE JOHN ROCKEFELLER McCORMICK MEMORIAL FUND
OF THE UNIVERSITY OF CHICAGO

THE UNIVERSITY OF CHICAGO PRESS
CHICAGO · ILLINOIS · U.S.A.

The Journal of Infectious Diseases

The Journal of Infectious Diseases is devoted to original investigations dealing with the general phenomena and special manifestations of infectious diseases. It is the aim of the journal to [illegible] and to include only such contributions as bear upon problems indicated in the title. The biology and chemistry of [illegible] organisms, the physiology and anatomy of the morbid processes [illegible], and the hygienic and sanitary problems to which they give rise [illegible] to be especially within the scope of the undertaking.

This journal was established in connection with the [illegible] Memorial Institute for Infectious Diseases, [illegible] of the late Mrs. Edith Rockefeller McCormick and the late [illegible] McCormick. Since 1941 it has been published in connection with the [illegible] McCormick Memorial Fund of the University of Chicago.

The Journal of Infectious Diseases is published by the University of Chicago Press, 5750 Ellis Avenue, Chicago, Illinois. The subscription price is $5.00 a volume; the price of single copies of current volume is $2.00. ¶ Remittances should be made on all orders from the United States, Mexico, Cuba, Puerto Rico, [illegible], Dominican Republic, Canary Islands, El Salvador, [illegible], Costa Rica, Ecuador, Guatemala, Honduras, Nicaragua, [illegible], Venezuela, Balearic Islands, Spain and Venezuela. ¶ [illegible] for Canada and Newfoundland, 40 cents on annual subscriptions [illegible] (total $2.10); for all other countries in the Postal Union, 75 cents [illegible] (total $2.75); on single copies 15 cents (total $2.15). ¶ Payments [illegible] payable to the University Press in postal or express money orders or bank drafts.

The following is an authorized agent:

For the British Empire, except North America, and [illegible] Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1. [illegible] of yearly subscriptions and on single copies may be had on application.

Claims for missing numbers should be made within [illegible] month of publication. The publishers expect to supply [illegible] but cannot undertake to replace copies [illegible] in transit and when the reserve stock will permit.

Business correspondence should be addressed to The University of Chicago Press, 5750 Ellis Avenue, Chicago 37, Illinois.

Communications for the editors and manuscripts should be addressed to Miss Dorothy W. Green, Assistant to the Editor, The Journal of Infectious Diseases, [illegible], Chicago 37, Illinois.

Entered as second-class matter August 1, 1913, at the post office at Chicago, Illinois, under the Act of March 3, 1879.

Acceptance for mailing at special rate of postage provided for in Section 1103, Act of October 3, 1917, Section 1103, amended February 28, 1925, authorized [illegible].

Notice to Subscribers: If you change your address, please notify the Circulation Department immediately. The Post Office does not forward second-class matter.

J. Infect. Dis. Vol. 92, No. 2

THE MIGRATION, DISTRIBUTION IN THE BRAIN, AND GROWTH OF ASCARID LARVAE IN RODENTS

JACK D. TINER

From the Department of Zoology, University of Illinois, Urbana, Illinois

This paper reports experiments on *Ascaris columnaris* of striped skunks, on an *Ascaris* from middle-western raccoons, on another *Ascaris* from the badger, on *Ascaris laevis* from Alaskan marmots, and on *Toxocara canis* of dogs. Central nervous system damage in rodents caused by the occurrence of ascaris larvae in the brain and spinal cord has been reported (Tiner, 1949 and 1951). The ascarid most pathogenic for the central nervous system of rodents is from the raccoon. Sprent (1950 and 1952) has experimented with *A. columnaris* and a variety of other ascarids, but to minimize unnecessary duplication in our studies, he did not work extensively on larvae in the brain or on the raccoon ascarid.

The writer (1949) reported briefly on the finding of larvae from skunk and raccoon *Ascaris* in intermediate hosts, and he (1951) indicated that the ascarids from these hosts have respectively different larval behaviors in rodents. Sprent (1952) has shown that though the name *A. columnaris* applies to the skunk ascarid, it is not appropriate for the species in the fisher and marten, which he named *Ascaris devosi*. He (1952) has also contributed much information on the abundance of ascarid larvae in the tissues of experimental mice for the first 28 days after infecting. All ascarids from carnivores which have been tested will encapsulate in rodents. Raccoon, skunk, and marten ascarids can become established in final hosts when rodents containing encapsulated ascarids are eaten (Tiner, 1949 and 1952; Sprent, 1952).

Received for publication July 10, 1952.

This work was supported by a Grant in Aid from the New York Zoological Society's 1951 Research Program at the Jackson Hole Research Station, Moran, Wyoming. Results of this study were presented to the Graduate School of the University of Illinois in partial fulfillment of the requirements for the Degree of Doctor of Philosophy. Thanks are expressed to Professor L. J. Thomas for his interest and for making laboratory space available.

METHODS

Ascarid eggs used in this study were taken from the uteri of adult female worms, or were harvested from fecal suspensions by sedimentation and flotation on saturated sodium chloride solution, and were incubated for at least 17 days in 1% formalin at room temperature before they were administered to experimental rodents, usually white mice. Either before or after incubation, the albuminoid coats of the eggs were removed with 2% sodium hydroxide and 0.01% sodium hypochlorite solution to slow the sedimentation rate of the eggs and thereby improve the uniformity of the suspension used for dosing. The laboratory mice to which larvae were fed had been given to the writer by Dr. B. V. Hall. Most of the mice had been used only in negative tumor transplant tests. However, the mice available at the time that growth curves were determined had survived LD_{50} total body exposure to X rays administered about 3 weeks before they were dosed with ascarid eggs.

Mouse craniums were opened after the animals had been killed with chloroform or ether. Portions of the brain and cervical spinal cord were removed with scissors and pressed between glass into a film of tissue about 50 μ thick. Parts examined usually included the cervical spinal cord, the medulla, the cerebellum, and the "cerebral region." This last-named portion consisted of all parts anterior to a vertical cut between the optic lobes and the cerebellum. Larvae found in the pressed-out brain tissue were traced with a camera lucida and measured.

RESULTS AND DISCUSSION

1. Migration of ascarid larvae fed to rodents

The sites of frequent encapsulation or localization in mouse tissues that are peculiar to skunk, marmot, raccoon, and badger ascarids can be interpreted as "milestones" along or near the migration pathway.

The next five paragraphs consist of impressions gained and observations made by the writer during the examination of about 500 experimental rodents during the past few years. Interpretation and discussion of these results conclude section 1.

The skunk ascarid, *A. columnaris*, encapsulates abundantly on the caecum and along the colon. Such encapsulated larvae, representing about 10% of the total dose given, apparently had penetrated the wall of the large intestine and remained in its musculature or beneath the serosa. Other skunk ascarid larvae complete a migration via the liver to the lungs, and from there many go out in the blood stream to the skeletal muscles and occasionally to the brain.

The marmot ascarid, *A. laevis*, penetrates the intestinal wall and travels to the liver, where lesions can be observed beneath the capsule on the 4th day. At this time a few tiny pulmonary hemorrhage spots are present. The number of these petechial hemorrhages in the lungs increases from the 5th to 7th days after *A. laevis* larvae have been fed. At least some of the liver lesions remain and become foci of necrosis in which nonencapsulated larvae freely move. These foci apparently did not interconnect as each one contained a living larva. The marmot ascarid is the only ascarid which the writer has observed to produce these chronic liver lesions and to escape encapsulation. It is also true that other species of nematode larvae which happen to localize in the brain are almost never walled off, but this is because brain tissue does not tend to form fibrous connective tissue capsules. The marmot ascarid has not been seen in tissues other than liver and lungs of experimental mice.

The badger ascarid sometimes encapsulated abundantly in the mesentery of the small intestine of *Peromyscus maniculatus artemisiae*. It frequently encapsulated on the epicardial surfaces of this mouse. Slicing of the heart into 1-mm thick sections usually failed to reveal additional cysts in the myocardium or adjacent to the endocardium. Ground squirrels, *Citellus armatus*, fed badger ascarids usually had similarly distributed encapsulations on the surface of the heart. They also had numerous capsules beneath the parietal pleura and on the diaphragm. This last mentioned tissue often had more larvae than any other part of the thorax, suggesting that larvae had reached it from the abdominal side as well as through the pleura. Ascarid larvae do not penetrate far into striated muscle. They are usually found encapsulated just beneath the parietal pleura and in the epicardium.

The distribution of raccoon ascarid cysts in tree squirrels (*Sciurus carolinensis* experimentally infected and *Sciurus niger* naturally infected) is particularly revealing of the wandering tendencies of migrating larvae. As mentioned previously (Tiner, 1949), these larvae are in the thorax. Their most typical localization site is the walls of the caval veins, which they must have reached by one of two possible routes—either from the lumina of the veins, or by wandering from other organs in the thoracic cavity to the perivascular tissues. Regarding the first possibility, it is very doubtful that larvae could ever

penetrate from the lumina. The diameter of these veins is great as compared to larval length, and it is improbable that the blood would be sufficiently viscous or stationary to furnish traction for the larvae to actively penetrate. Furthermore, the larvae encyst on anterior and posterior caval veins, and on the dorsal and ventral sides of the veins in approximately equal numbers. Should larvae be penetrating from the lumina of the veins, we would expect to find a larger number of cysts in the wall of the postcava, rather than the precava.

The second possibility has evidence in its favor. Badger ascarid larvae have been found widely distributed in the skeletal muscles of *Citellus armatus*, but they are most abundant in the intercostal spaces just under the parietal pleura and in the diaphragm. Similarly located raccoon ascarid larvae have been found in infected tree squirrels. Both raccoon and badger ascarid larvae have been found on the external surface of the heart. These data suggest that carnivore ascarid larvae are able to leave the lungs by penetrating pleural surfaces and crossing the pleural cavity.

Any interpretation that ascarid larvae are able to leave the lungs by wandering into adjacent tissues necessarily suggests that such larvae also wander about in the lungs. Raccoon and badger ascarid larvae are like *A. columnaris* larvae in that they reach the lungs very rapidly (see Sprent, 1952). Random wandering in lung tissue could easily take them into a venule, whence they would be carried back to the heart and out into the peripheral tissues with the arterial blood. This would account for the somatic migration that Sprent (1952) has found to be typical for larval ascarids from terrestrial carnivores. Chance wandering would be expected to take some larvae into respiratory bronchioles, and eventually onto the ciliated epithelium of the bronchi which would carry them up the trachea to the digestive tract. This chance entry into bronchioles could very well account for some of the smaller fraction (about one-fifth) of the *A. columnaris* and *Ascaris mustelarum* (now *A. devosi*) larvae which Sprent (1952) has shown to be lost via the intestine of mice.

The greater size of *Ascaris lumbricoides* larvae reaching the lungs after a period of growth in the liver would also make their re-entry into the circulatory system less probable. Sprent's (1952) charts show that there is a shift of *A. columnaris* and *A. devosi* larvae from the lungs to the peripheral tissues, and that this process begins almost immediately. Apparently it does not terminate until the end of the 2nd week. If the growth curve for the skunk ascarid applies to larvae in the lungs of mice as well as in brain tissue, then there is a possibility that larvae of minimal length (less than 0.4 mm long) are still leaving the lungs after 10 days. Such a shift of these smaller larvae could account for the decrease of larvae in the lungs and the increase of larvae in the peripheral tissues.

2. Distribution of larvae in the brain

Early in the investigation it was noticed that skunk or raccoon ascarid-infected mice tended to have more larvae in the medulla than in any other part of the brain. Although this portion represented only one-fifth of the total mass, it often contained half the larvae present in the brain. This concentration of larvae in the medulla prevented answering the question of whether a mouse would be affected by a single larva in the cerebellum, diencephalon, or cerebrum. Mice sacrificed at 10 days frequently had larvae in the cerebrum and cerebellum, or in both the medulla

and in the other three portions into which the brain was divided.

An experiment was set up to determine the symptoms produced by a single larva in the cerebellum or cerebrum. Low dosages of raccoon ascarid larvae (about 28 per mouse) were given, and a set of 18 controls was killed and examined at 11 days in order to make sure that "cerebral" infections had been produced in the absence of other larvae in the brain. Brains of 8 mice contained no larvae, while those in the other 10 were distributed as follows: Five mice had a total of 6 larvae present in the cerebral portions but none in the medulla. In addition, 4 other mice had larvae in both the medulla (5 larvae) and in the cerebral portions (1 larva each, or 4). At 22 days the 30 remaining mice were killed and frozen. Those mice showing symptoms were each tagged with a list of their symptoms and placed in a large envelope. Mice showing no symptoms were placed in another envelope, and each group was weighed. The 14 healthy mice weighed 440 g, whereas the net weight of the 16 ailing mice was 412 g. The diseased animals were smaller and had rough hair coats. In every instance it had been possible to distinguish the diseased from the healthy mice by their symptoms of nervous system impairment and poorer appearance. Examination of the two groups of mice showed that none of the healthy mice had larvae in the brain, whereas all those in the ailing group had larvae in their brains. Furthermore, all the latter mice had larvae in the medulla and cervical spinal cord (19 and 4 larvae, respectively). Except for a larva in the mesencephalon of one mouse, the medulla and spinal cord were the only regions containing larvae.

The most likely explanation for this shift in larval abundance seems to be a migration of the larvae into the medulla from parts of the brain anterior and dorsal to it. Evidence that *Setaria* larvae wander in the brains of affected herbivores (see Innes et al, 1952) would seem to add weight to this interpretation.

Additional larvae might reach the peripheral tissues from the lungs after 10 days, while a higher return rate from some of these tissues to the lungs would give the appearance of a migration into adjacent parts. Badger ascarid larvae are abundant in the brain of heavily infected *Peromyscus maniculatus* at 3 days. They disappear from the brain by the 10th day but remain abundant in the masseter muscles.

The writer has observed occasional raccoon ascarid larvae coated over by host resistance mechanisms (precipitates?) on the 7th day. Opaque spots where skunk ascarid larvae must once have been were seen in some mouse brains. The numbers of these spots often exceeded the total number of larvae persisting in the same brain. Occasional raccoon and skunk ascarid larvae have been found dead in the brain. These observed instances of host resistance are exceptions, however. Most of the larvae of either ascarid in the mouse brain are alive and in good condition when the larvae are large enough to be found readily. Host resistance might destroy larvae in the early days of infection when the larvae are small and not readily observed, or the rate of disintegration of larvae which are attacked after 10 days could be very high so that they are rarely seen. All the other possibilities considered here seem to be auxiliary mechanisms, at best, to the probable direct posteriad migration of larvae through brain tissue to the medulla and spinal cord.

If a similar shift in larval abundance occurs in *Peromyscus*, then the phenomenon is important in the epidemiol-

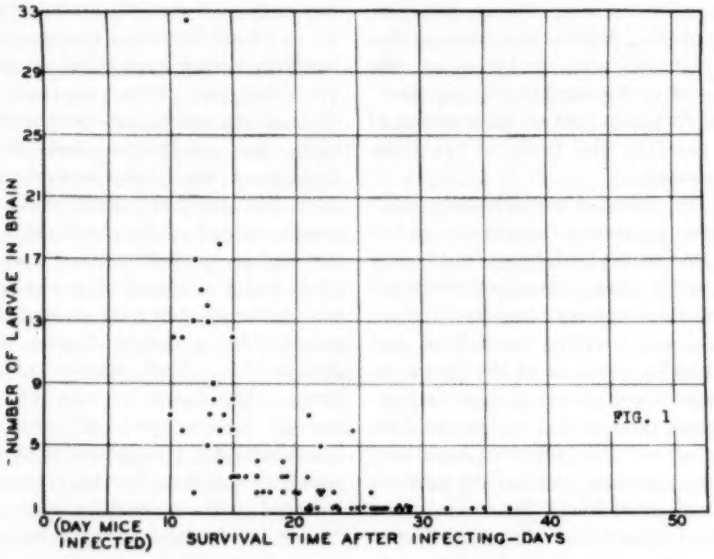
ogy of raccoon and skunk ascarids. The writer's (1951) statement that "one raccoon ascarid larva in the medulla or spinal cord of mice is fatal" referred to about half of the number of larvae reaching the brain of the laboratory mouse.

Mice fed raccoon ascarids have most often been examined toward the end of the 2nd week after infecting, and larvae were usually about equally distributed between the spinal cord and medulla, on the one hand, and the cerebellum and more anterior portions of the brain, on the other. Various low dosage experiments have proven that before the 20th day almost all the larvae in surviving mice have somehow reached the medulla and spinal cord. Under field conditions one would expect mice to be uninfected or to harbor only one or two larvae in the brain. If the shift in larval abundance were to take place in wild mice, then almost all those with larvae in the brain would be killed, rather than only that group (half or less) of the mice which originally received larvae in their medulla and/or spinal cord.

3. Growth of raccoon and skunk ascarid larvae

The growth and maximum larval size of raccoon, badger, skunk, and dog ascarid larvae in the rodent brain correlate with their respective pathogenicities. This observation was made on the above species in a variety of rodents, and verified by controlled experiments using each of the ascarids in albino mice and guinea pigs. The badger ascarid did not infect the guinea pig brain, but it was observed and measured in the brain of the ground squirrel, *Citellus armatus*. Larval size maxima were obtained for each of the ascarids in the brains of the rodents at various times after infecting. Enough data were obtained on raccoon and skunk ascarid larvae to plot growth curves (fig. 2 and 3), in which the mean lengths of larvae (ordinate) from mice killed on each day (abscissa) are plotted as open circles. Dots above and below these circles indicate the maximum and minimum lengths of the larvae measured. Mice used had survived irradiation experiments about 3 weeks previously. No difference in growth rates of larvae in these and in ordinary mice was detected, but the irradiated mice were at least as susceptible as ordinary mice to brain invasion by both species of ascarid larvae. The larger number of raccoon ascarid larvae per individual mouse brain made for greater uniformity of the experimental data for this species. Mice infected with several raccoon ascarid larvae in the brain almost never lived beyond the 15th day (see fig. 2). Lengths could not have been obtained after this date without using large numbers of lightly infected mice, most of which would have had 0, 1, or 2 larvae in the brain.

When the number of larvae in the brain (ordinate) and the day of death of nonirradiated mice (abscissa) were plotted (fig. 1 and 4), it was found that death did not occur before the larvae had grown to an average length of 1 mm (see fig. 2 and 3). This occurs on the 9th day for the raccoon ascarid, usually after the 25th day for the skunk ascarid, and has never been observed for *T. canis* from dogs. The maximum length of *T. canis* larvae in the rodent brain was less than 0.5 mm. Raccoon ascarid larvae in the viscera of fatally infected guinea pigs were not yet 1 mm long at the end of 2 weeks. This experiment was repeated and for the second time almost all larvae in the guinea pig viscera were less than 1 mm long. The average length of larvae in the brains of these same animals was 1.3 mm. The badger ascarid has been measured extensively



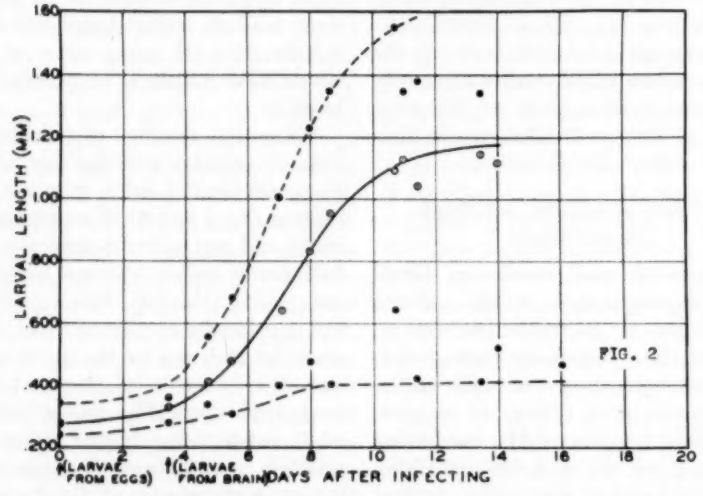
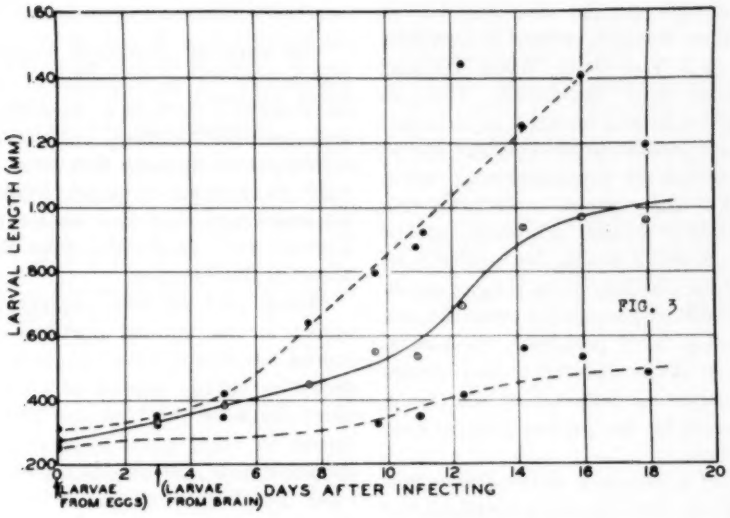


FIG. 1.—The relationship between the number of raccoon ascarid larvae in the brain and the survival time of mice after infection.

Each dot shows the number of larvae in a single mouse brain and the day of death of the animal.

FIG. 2.—Growth of raccoon ascarid larvae in mouse brain.

Measurements were made of larvae broken from eggs and of larvae in groups of mice killed on varying days after infecting. Average lengths for each day are plotted as open circles and are connected with a solid line. Maximum and minimum larval lengths for each day are plotted above and below, respectively, and are connected with dash lines.



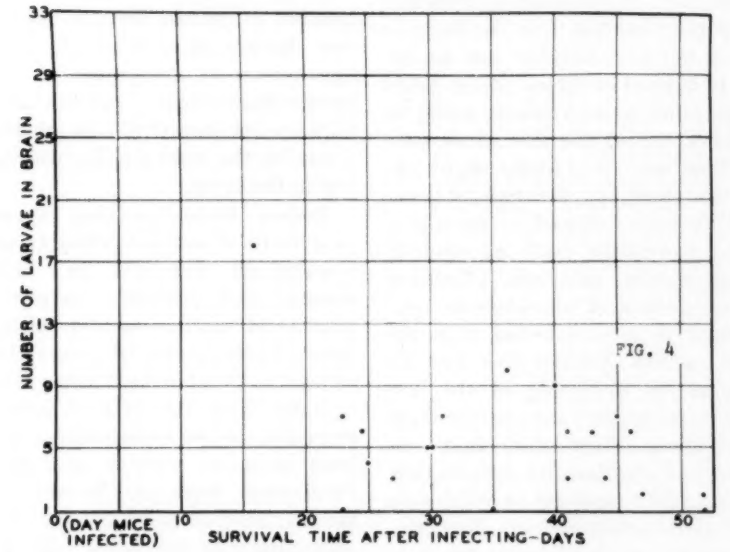


FIG. 3.—Growth of skunk ascarid larvae in mouse brain. For explanation, see figure 1.

FIG. 4.—The relationship between the number of skunk ascarid larvae in the brain and the survival time of mice after infection.

For explanation, see figure 3.

only in the central nervous system of *Citellus armatus*, where it averaged about 1.3 mm long when obvious symptoms had developed. Two to five larvae in the medulla of this animal were insufficient to produce symptoms in the 21 days following infection that the animals were observed, but 9 larvae caused symptoms to appear in about 2 weeks. No tendency of badger ascarid larvae to congregate in the medulla of the ground squirrel brain was noted. Very probably, the size of the brain of the infected rodent species is important in determining whether a few ascarid larvae are likely to produce symptoms.

Day of appearance of the first symptoms would also be worth plotting, but these are not easily detected until one is thoroughly familiar with the limits in which a mouse's activity can be ascribed to normal behavior. Many hours of observation of each mouse would be required to detect the first abnormalities. These were frequently slight unsteadiness, trembling, slowness, or lameness. They were followed by more pronounced symptoms such as circling, spinning, rolling, paralysis, blindness, and many others. A transition to coma came next in mice infected with the raccoon ascarid. Usually this was accompanied by worsening of the first symptoms noted, and then two or three days of coma. Most often death resulted from starvation and dehydration. Skunk ascarid infections in mice were similar, and sometimes fatal. However, initial symptoms persisted indefinitely without growing worse in some mice, and even disappeared in others. Mice infected with several skunk ascarid larvae in the brain did not necessarily become ill. As Sprent (1952) has indicated, permanent symptoms begin appearing about the 17th day rather than on the 7th to 10th days noted by the writer for the raccoon ascarid.

SUMMARY AND CONCLUSIONS

The sites of abundant localization that result for each species of ascarid larva studied were on or near the common ascarid migration pathway. This is interpreted to mean that some larvae tend to wander outward from each visceral organ that they pass through. Larvae very probably also wander about in these organs.

Skunk and raccoon ascarid larvae arrive in the various portions of the mouse brain in numbers roughly corresponding to the masses of these portions. Between the 10th and 20th days larvae in other parts appear to have migrated into the medulla and spinal cord. The number of larvae in the last two regions increases so that the total number of central nervous system larvae changes little, if at all. The first appearance of symptoms of nervous system impairment in rodents correlates with the attainment of a length of about 1 mm by the most rapidly growing larvae in the brain.

Rodent brain-damaging larvae appear to be of survival value to ascarid populations parasitic in raccoons, skunks, and probably badgers. The number of raccoon ascarid larvae persisting in the tissues of most rodents is as high as in laboratory mice, if we are to judge from the data on field infections and the few experiments that have been done on captive squirrels and *Peromyscus leucopus*. Rodent central nervous system damage appears to be associated with the ordinary life history of the raccoon ascarid. Larvae causing such damage serve to increase the probability that the intermediate host will be caught by the final host. We do not know how raccoons fitted into the primeval North American forest which contained a variety of predators. At present they tend to dominate large lowland woodlots in the north central

states. In these particular environments, the chances that an infected intermediate host will be eaten by a raccoon may be greater than its being consumed by any other species.

REFERENCES

Innes, J. R. M., Shoho, C. and Pillai, C. P. 1952, Epizootic cerebro-spinal nematodiasis or setariasis. Brit. Vet. J. **108**: 71–88.

Sprent, J. F. A. 1950, Observations on the life history of *Ascaris columnaris*. J. Parasitol. **36** (Supplement): 29.

———. 1952, On the migratory behavior of the larvae of various ascaris species in white mice. J. Infect. Dis. **90**: 165–176.

Tiner, J. D. 1949, Preliminary observations on the life history of *Ascaris columnaris*. J. Parasitol. **35** (Supplement): 13. No. 6.

———. 1951, Observations on larval carnivore ascarids in rodents. J. Parasitol. **37** (Supplement): 21–22.

———. 1953, Fatal central nervous system disturbances in rodents produced by *Ascaris* larvae, and the epidemiology of these nematodes in carnivores and rodents. J. Mammal. **34**: In press.

ON THE MIGRATORY BEHAVIOR OF THE LARVAE OF VARIOUS ASCARIS SPECIES IN WHITE MICE

II. LONGEVITY OF ENCAPSULATED LARVAE AND THEIR RESISTANCE TO FREEZING AND PUTREFACTION

J. F. A. SPRENT*

From the Department of Parasitology, Ontario Research Foundation, Toronto, Canada

It has been demonstrated that certain ascaris parasites of carnivorous mammals, namely *Ascaris columnaris* from the skunk (Tiner, 1949) and *Ascaris devosi* from the marten (Sprent, in press) utilize an intermediate host in the course of their life history. Infection of the final host occurs through the ingestion of larvae encapsulated within the tissues of rodents. Encapsulation of larvae in the tissues of rodents has also been demonstrated for *Toxocara canis* (Fülleborn, 1921), *Toxascaris leonina* (Wright, 1935) and *Toxascaris transfuga* (Sprent, 1951). It has been shown (Sprent, 1952) that the larvae of these three species may remain alive for at least 4 weeks in the tissues of mice, but it has not been shown that infection of the final host may occur through ingestion of mice. This method of infection has been suggested for *T. canis* by Fülleborn (1921) and by Hoeppli, Feng and Li (1949). Hoeppli et al observed that the larvae of *T. canis* frequently become encapsulated in the liver of mice and that they remain alive there for many weeks. Beaver et al (1952) reported that the larvae of *T. canis* and *Toxocara mystax* remain alive in various tissues of mice for several months. Sprent (unpublished) has recovered fourth stage larvae of *T. canis* from the intestine of young dogs fed infected mice 8 days previously.

The experiments reported in the present paper were designed to show the following: (1) the distribution of larvae in the various tissues of mice over periods longer than 4 weeks, and (2) the length of time the larvae will remain alive in the tissues of mice. In addition, some observations are recorded on the resistance of larvae to freezing and putrefaction.

METHODS

The methods used for collection of eggs, for infection of mice and for the recovery of larvae from the tissues were as previously described (Sprent, 1952). The mice in these experiments were kept for periods up to 6 months and killed at intervals as shown in table 1. The various component tissues shown in this table were separated and subjected to emulsification and digestion. The mesenteric tissues included the mesentery, removed by tearing along the length of the intestine, and its associated lymph nodes and fat. The spleen, rectum, feet and fur were discarded. After the removal of the above and the tissues and organs listed in the table, the carcass was divided into three components, namely the head and the front and back of the trunk, by cutting behind the head and at the junction of the dorsal and lumbar vertebrae.

EXPERIMENTAL

Distribution of larvae in mouse tissues (1 to 6 months).—The distribution of larvae in the tissues of the mice was estimated and is shown in table 1. Larvae counts were made after preservation, but before adding the preservative the condition of the larvae in the carcasses was noted and in all cases they were found to be vigorously motile.

Received for publication July 28, 1952.

* Present address: University of Queensland, The Veterinary School, Brisbane, Australia.

This work was supported by the Province of Ontario on the recommendation of the Research Council of Ontario.

It was evident that the larvae of the ascaris parasites of carnivores remain alive in the tissues of white mice for at least 6 months. The distribution of larvae was not followed beyond this time but mice were kept up to a year after infection with *T. transfuga* and living larvae were still recovered from their tissues.

The relative distribution of the larvae in the various tissues was remarkably similar to that already reported (Sprent, 1952). In all species most of the larvae were distributed in the head and carcass i.e., the general musculature of the body. In the case of *A. columnaris* many larvae were encapsulated in the liver, lungs and heart, while in the case of *A. devosi* larvae were less often found in these organs. In the case of *T. canis* the larvae were found frequently in the brain but appeared to cause no disturbances of locomotion. This was in marked contrast to infection with larvae of *A. columnaris*, in which such disturbances are frequently observed in mice. The larvae of *T. transfuga* were found in relatively large numbers in the wall of the intestine and in the mesenteric tissues; this was in agreement with previous findings in which *T. leonina* and *T. transfuga* were found to be closely related in this respect (Sprent, 1952).

Resistance of encapsulated larvae to cold temperatures.—Mice were infected with eggs of various ascaris species and were killed at various intervals after infection. The bodies were immediately placed in a refrigeration unit at −20 C. They were removed after various intervals and were digested for 3 hours to recover the larvae. The motility of the recovered larvae was noted after digestion and the results are shown in table 2. It will be observed in the case of *A. columnaris* and *A. devosi* that where the duration of infection of the mouse was more than about 3 weeks the larvae recovered vigorous motility after a sojourn of several weeks at −20 C. Larvae from shorter periods of infection were less resistant. It appears likely that the resistance of the larvae de-

TABLE 1.—*Distribution of ascaris larvae in tissues of mice (1 to 6 months after infection).*

	Dose of eggs	Duration of infection (months)	Heart	Lungs	Liver	Mesenteric tissues	Brain	Kidney	Intestinal wall	Carcass		
										Head	Front	Back
A. columnaris	5000	1	60	10	80	0	10	10	0	230	720	150
		2	2	8	42	2	0	0	0	152	1350	620
		3	40	12	80	1	0	0	8	270	670	160
		4	16	10	385	30	0	5	55	200	1070	130
		6	10	15	100	60	0	5	40	250	200	95
A. devosi	5000	1	4	0	0	0	0	1	0	120	2240	340
		2	0	8	0	0	0	0	0	254	2100	252
		3	4	6	15	0	0	0	10	220	1400	160
		4	0	0	0	0	0	0	0	140	1640	340
		6	10	10	0	0	0	0	0	240	2500	160
T. mystax	2500	1	0	0	0	0	0	10	0	100	300	430
		2	0	0	0	0	0	0	30	110	380	240
T. canis	2500	1	0	10	10	0	200	10	0	160	160	140
		2	0	0	0	2	0	0	0	166	74	44
		3	0	0	0	0	90	0	0	120	300	180
		4	0	0	10	0	30	0	0	340	110	158
		6	0	0	0	0	170	0	0	140	100	90
T. transfuga	5000	1	0	80	40	250	0	0	160	40	50	30
		2	14	6	0	150	14	0	138	212	510	450
		3	6	38	0	110	4	0	415	60	140	70
		4	0	70	0	260	0	0	250	70	180	180
		6	50	0	0	320	0	0	207	160	560	120

Each line of data is for a single mouse.

TABLE 2.—*Resistance of encapsulated larvae of various ascaris species to low temperature.*

Species	Interval between infection and death of mouse	Duration of exposure (−20 C)	Motility of larvae
		Weeks	
A. columnaris	3 weeks	8	++
	4 weeks	8	++
	5 weeks	8	++
	6 weeks	8	++
	6 weeks	12	++
	6 weeks	14	++
	6 weeks	15	++
	6 weeks	16	++
	6 weeks	17	++
	6 weeks	18	++
A. devosi	4 days	10	±
	5 days	5	+
	6 days	5	+
	10 days	4	+
	19 days	3	++
	15 weeks	4	++
	15 weeks	8	++
T. canis	8 days	6	−
	8 weeks	4	++
T. transfuga	6 days	6	−
	7 weeks	12	−
	9 weeks	16	−
	6 months	5	+
	9 months	16 days	++

Each line of data is for a single mouse.
++ Most of the larvae vigorously motile.
+ Some larvae motile.
± Slight movement observed.
− Apparently dead.

pends upon the capsule in which they lie. These were not conspicuous until 2 to 3 weeks after infection. The larvae of *T. transfuga* were evidently less resistant than the former two species.

Resistance of encapsulated larvae to putrefaction.—The bodies of infected mice were allowed to undergo putrefaction at summer room temperature (80 F). At various intervals the mice were digested and the motility of the recovered larvae was observed (table 3). Encapsulated larvae of *A. columnaris*, *A. devosi* and *T. transfuga* are evidently able to withstand putrefaction for at least 6 days.

TABLE 3.—*Resistance of larvae of various ascaris species to putrefaction of the infected mouse.*

Species	Interval between infection and death of mouse (weeks)	Duration of putrefaction at 80 F	Motility of larvae*
A. columnaris	12	2	++
A. columnaris	12	4	++
A. columnaris	12	6	++
A. columnaris	12	7	±
A. columnaris	12	10	−
A. devosi	8	6	++
T. canis	16	6	−
T. transfuga	8	6	++

Each line of data is for a single mouse.
* See table 2.

DISCUSSION

The results show that the larvae of *A. devosi* and *A. columnaris* when encapsulated in the tissues of mice appear to be well located for reaching their final hosts under natural conditions. Firstly, they are able to survive for long periods in the tissues of the mouse, suggesting that many mice in the vicinity of the natural host would be infected. Secondly, most of the larvae are encapsulated in the anterior end of the mouse's body; the Mustelidae frequently begin by eating the head and neck of their prey and may discard the rest of the carcass. Thirdly, it seems likely that infection may occur even if some time has elapsed between death of the rodent and the ingestion of its carcass. It seems probable that the larvae might remain alive in the carcass throughout the whole winter. In the event of the mouse dying in the summer months several days might elapse before the carcass is found and eaten; nevertheless the larvae may still remain infective, though putrefaction may be advanced.

SUMMARY

The distribution of the larvae of *Ascaris columnaris*, *Ascaris devosi*, *Toxocara canis* and *Toxascaris transfuga* was estimated in the tissues of mice over periods from 1 to 6 months after infection. It was found that most of the larvae of all species were distributed to the general musculature. It was observed in the case of *A. columnaris* and *A. devosi* that the larvae were particularly concentrated in the cervical and thoracic regions. In the case of *T. transfuga* a

high proportion were encapsulated in the intestinal wall and mesenteric tissues. In the case of *T. canis* a relatively high proportion of the larvae were recovered from the brain. The larvae of all species were vigorously motile after being in the tissues for 6 months.

The larvae of *A. columnaris*, *A. devosi* and *T. canis* recovered vigorous motility after digestion of infected mouse carcasses kept at − 20 C. for several weeks. The larvae of *T. transfuga* appeared to be less resistant to cold. The resistance of the larvae to cold appeared to develop about 3 weeks after infection. The encapsulated larvae of *A. columnaris*, *A. devosi* and *T. transfuga* were still vigorously motile 6 days after putrefaction of the mouse's body at room temperature.

REFERENCES

Beaver, P. C., Snyder, C. H., Carrera, G. M., Dent, J. H. and Lafferty, J. W. 1952, Pediatrics, **9**: 7–19.

Fülleborn, F. 1921, Arch. f. Schiffs- u. Tropen-Hyg. **25**: 367–375.

Hoeppli, R., Feng, L. C. and Li, F. 1949, Peking Nat. Hist. Bull. **18**: 119–132.

Wright, W. H. 1935, Proc. Helm. Soc. Wash. **2**: 56.

Sprent, J. F. A. 1951, J. Parasitol. **37**: 326–327.

———. 1952, J. Infect. Dis. **90**: 165–176.

———. Parasitology, In press.

Tiner, J. D. 1949, J. Parasitol. **35**: suppl. p. 13.

INCIDENCE OF ANTIBODIES FOR TOXOPLASMA AMONG VARIOUS ANIMAL SPECIES

LOUISE T. MILLER AND HARRY A. FELDMAN

The availability of the dye test,[1] a relatively simple but sensitive method for detecting antibodies for toxoplasma, permits surveys for serological evidence of infection by this parasite to be conducted with relative ease. In this report are presented the results of such antibody determinations among 298 members of 6 animal species. The purpose of this survey was to ascertain the importance of these animals as reservoirs for the human disease.

MATERIALS AND METHODS

Animal serums.—The animals were all adult and laboratory bred, except for the dogs and cats,* which were strays, and the bovine serums,* which were obtained from experimental herds. Samples from 54 albino rats, 76 cattle, 44 cats, 51 dogs, 51 guinea pigs and 22 rabbits were included in the study.

Serological method.—Antigen was obtained from 3- or 4-day peritoneal exudate prepared by inoculating albino mice intraperitoneally with the RH strain of toxoplasma.[2] All test serums were inactivated for 30 minutes at 56 C, and their antibody content was measured by the dye test method of Sabin and Feldman.[1]

RESULTS

The incidence of antibodies for toxoplasma among the various animals is summarized in table 1. It will be noted that none of the laboratory-bred rats was found to be positive. This is in contrast with the observations in the rabbit and guinea pig, both of which are also caged, laboratory-bred animals. One rabbit's serum was positive in a titer of 1:16 while 21 were negative. Although 32 of the 51 guinea pig serums were negative, 2 were positive undiluted, 3 at 1:4, and 14 had titers ranging from 1:16 to 1:4096. For experimental reasons the 2 herds of cows have been maintained as separate groups for many years. Of the 66 specimens from the first herd, 37 were negative, 20 were positive undiluted and 9 at 1:4. All 10 serums from the second herd were negative.

Among 44 cat serums,† 20 were definitely negative, while 3 were positive undiluted, 6 each at 1:4 and 1:16 and 9 at 1:64.

Received for publication August 7, 1952.

From the Dept. of Medicine, State University of New York Medical Center at Syracuse and the Kilian Research Laboratory, Wieting-Johnson Hospital.

This study was supported principally by a grant from the National Institutes of Health, U. S. Public Health Service, Bethesda, Maryland and, in part, by funds supplied by the Masonic Foundation for Medical Research and Human Welfare.

We are also indebted to the Hendricks Research Fund for aid in purchasing certain equipment.

Based upon a study submitted by Louise T. Miller in partial fulfillment of the requirements for the Master of Science Degree, Syracuse University, Syracuse, N. Y.

* We are indebted to Dr. Jean Holzworth of the Angell Memorial Hospital, Boston, Mass. for the cat serums and to Dr. James A. Baker of the College of Veterinary Medicine, Cornell University, for the cattle serums. The dogs were supplied by Dr. Gordon Moe of the Dept. of Physiology, the rats by Dr. Tepperman and Dr. Bass of the Dept. of Pharmacology, the guinea pigs by Dr. O. D. Chapman of the Dept. of Bacteriology and the rabbits by Dr. P. A. Bunn of the Dept. of Medicine of this College of Medicine.

1. Sabin, A. B. and Feldman, H. A. 1948, Dyes as microchemical indicators of a new immunity phenomenon affecting a protozoon parasite (toxoplasma). Science, **108:** 660–663.
2. Sabin, A. B. 1941, Toxoplasma encephalitis in children, J.A.M.A. **116:** 801–807.

† Serums from 9 additional cats were studied at a later date. Six of these were negative, 1 was positive at 1:4, 1 at 1:16 and the last titered to 1:256.

TABLE 1.—*Incidence of antibodies for toxoplasma among six animal species.*

Species	Number tested	Negative	Titers of positive reactors (dye test)						
			Undiluted	1:4	1:16	1:64	1:256	1:1024	1:4096
Albino rat	54	54	0	0	0	0	0	0	0
Cattle									
Herd 1	66	37	20	9	0	0	0	0	0
Herd 2	10	10	0	0	0	0	0	0	0
Rabbit	22	21	0	0	1	0	0	0	0
Cat	44	20	3	6	6	9	0	0	0
Dog	51	17	2	2	9	11	8	2	0
Guinea pig	51	32	2	3	2	4	3	3	2
Totals	298	191	27	20	18	24	11	5	2

Of the 51 dog serums, 17 were negative, 2 each were positive undiluted and at 1:4, while 20 had titers ranging from 1:16 and 1:1024.

DISCUSSION

The importance of low dye test titers remains to be determined. We believe that positive tests which are limited to the undiluted specimen are without significance and may be classed as negative. Serums which are positive in a 1:4 dilution are possibly doubtful and consequently were considered with the negative specimens. Titers of 1:16 are readily reproducible and we considered these and higher titers to be indicative of past or current infection.

An interesting discrepancy exists among the results obtained in the serums of the laboratory-bred animals: where the rat serums were all negative, one of 22 rabbits (4.5%) and 14 of 51 guinea pigs (28%) were positive. Although we have no explanation for these serological differences, it is conceivable that the manner in which such animals ordinarily are housed is an important factor. Rabbits and rats tend to be maintained either singly or in pairs, but guinea pigs are likely to be quartered in larger groups. Thus animal-to-animal transmission is more likely to occur among guinea pigs than among either of the other two species. It is also evident from these data that neither the guinea pig nor the rabbit should be used as the host in which to attempt to isolate toxoplasma from other sources.

Our results with the dye test agree with Sabin's study[3] in which he found that 5% of laboratory rabbits were immune to toxoplasma when examined with the rabbit neutralization test. Christiansen and Siim,[4] on the other hand, surveyed the wild hare population of Denmark and found that 9.4% of the animals had antibodies for toxoplasma. They found that this figure fluctuated with the seasons, being highest from January through March.

On the basis of what has been said above, all of the cattle serums would have to be considered as negative. While these herds have been kept in separate quarters for some years, they are exposed to various insects and, probably, rodents as well. One might conclude from these data that cattle are not frequently infected with toxoplasma. If this is true then it is unlikely that cow's milk represents a significant hazard to humans.

Jacobs and Jones[5] and Grocott[6] dem-

3. Sabin, A. B. 1942, Toxoplasma neutralizing antibody in human beings and morbid conditions associated with it. Proc. Soc. Exper. Biol. & Med. **51**: 6–10.
4. Christiansen, M. and Siim, J. C. 1951, Toxoplasmosis in hares in Denmark. Serological identity of human and hare strains of *Toxoplasma*. Lancet, **1**: 1201–1203.
5. Jacobs, L. and Jones, F. E. 1950, The parasitemia in experimental toxoplasmosis. J. Infect. Dis. **81**: 79–89.
6. Grocott, R. G. 1950, A case of canine toxoplasmosis from the Canal Zone. Am. J. Trop. Med. **30**: 669–675.

onstrated toxoplasma in dog tissues. Since we found 59% of our dogs to have significant amounts of antibody it would seem that these reports[5,6] should be readily reproducible. In addition to the evidence presented for the frequent infection of the dog, 34% of the cats which we studied also had significant amounts of toxoplasma antibody. Since these two species are commonly associated with human dwellings, they may represent important reservoirs for human infections. The frequency of these positive serums complicates the problem of proving that a particular dog or cat is related to a given instance of human disease. For example, we have recently encountered two cases of congenital toxoplasmosis in families where, in one instance a pet cat, and in the other a pet dog, had been ill during the time of the mother's pregnancy. Although the serum of each animal had significant amounts of antibody we were unable to implicate either as having been important in the human infections because of the high incidence of antibodies among stray cats and dogs.

SUMMARY

Antibodies for toxoplasma were detected, with frequency, among street cats and dogs and laboratory-bred guinea pigs. They were not found at all among laboratory-bred rats and with slight frequency among laboratory-bred rabbits. A large number of cattle comprising one herd which had been maintained for some time for experimental purposes had antibody, but of such low concentration as to be of questionable significance.

ANTIGENIC AND CULTURAL RELATIONSHIPS OF *LACTOBACILLUS BIFIDUS* AND *LACTOBACILLUS PARABIFIDUS*

NED B. WILLIAMS, ROBERT F. NORRIS, AND PAUL GYÖRGY

From the Department of Microbiology, School of Dentistry; the William Pepper Laboratory of Clinical Medicine; and the Nutritional Laboratory, Department of Pediatrics, University of Pennsylvania, Philadelphia 4, Pennsylvania

For many years, the lack of a suitable medium retarded the study of *Lactobacillus bifidus*. In 1950, Norris, Flanders, Tomarelli, and György[1] reported that a modification of the Teply and Elvehjem medium[2] is satisfactory for the primary isolation and laboratory maintenance of *L. bifidus* as a branched microaerophilic organism. Even in this medium, *L. bifidus* may change abruptly, without apparent cause, to aerobic, unbranched bacilli which will then grow on many different mediums. If it were not for the fact that colonies of the two types of organisms are easily distinguished, it would be difficult to keep the branched organisms in pure culture. Because of these and other differences, Norris et al[1] proposed that the unbranched variants, or mutants, be called *Lactobacillus parabifidus* and the term *L. bifidus* be applied only to microaerophilic branched organisms. The terminology of Weiss and Rettger[3] and of Bergey's Manual[4] thus was reversed.

In a recent report, Hassinen, Durbin, Tomarelli, and Bernhart[5] found that four of the strains originally isolated in our laboratory, and maintained for about two years with transfers every second day, grew on a simple synthetic medium. In an effort to isolate new strains from feces, they reported that although strains of *L. bifidus* grew initially on this simplified medium, subcultures of these strains grew only on the modified Teply and Elvehjem medium.[1] This suggested that *L. bifidus* may occur as more than one type.

Reports on serologic reactions support the view that *L. bifidus* is not serologically homogeneous. Blaurock[6,7] reported that organisms from smooth and rough colonies were agglutinated only by the homologous antiserum and that four serologic types could be distinguished by means of agglutination tests. The findings reported by Boventer[8] were similar to those of Blaurock. Neither worker confirmed the serologic relationship between *L. bifidus* and *L. acidophilus* previously reported by Cruickshank[9] and Weiss and Rettger.[3] The discrep-

Received for publication August 28, 1952.

These studies were aided by a contract between the Office of Naval Research, Department of the Navy and University of Pennsylvania (NR 183-333), and by a grant from Wyeth Incorporated.

The authors wish to express their apprecation for technical assistance to Miss Eva Blau, Miss Nancy L. Minnick, Miss Annabelle Avery, and Mrs. Evelyn Feldbaum.

1. Norris, R. F., Flanders, T., Tomarelli, R. M. and György, P. 1950, J. Bact. **60**: 681–696.
2. Teply, L. J. and Elvehjem, C. A. 1945, J. Biol. Chem. **157**: 303–309.
3. Weiss, J. E. and Rettger, L. F. 1934, J. Bact. **28**: 501–521.
4. Breed, R. S., Murray, E. G. D. and Hitchins, A. P. 1948, Bergey's Manual of Determinative Bacteriology, ed. 6, Baltimore, Williams and Wilkins Co. pp. 350–361.
5. Hassinen, J. B., Durbin, G. T., Tomarelli, R. M. and Bernhart, F. W. 1951, J. Bact. **62**: 771–777.
6. Blaurock, G. 1937, Monatschr. f. Kinderh. **68**: 304–309.
7. Blaurock, G. 1939, Zentralbl. f. Bakt. **144**: 75–79.
8. Boventer, K. 1938, Zentralbl. f. Bakt. **142**: 419–430.
9. Cruickshank, R. 1925, J. Hyg. **24**: 241–254.

TABLE 1.—*Data on cultures and preparation of antiserums.*

Strain	Isolation date	Source*	Number of months to obtain antiserum	Number of courses used for each method of immunization				Total amount of vaccine in ml	
				A	B	C	D	Concentrated	Dilute
Jackson	4/14/49	BF	2½	3	2				10.5
Jackson M	12/12/49	M	1¼	2					2.0
Jackson S	1/12/50	M	3				1 i.p. 3 i.v.	1.0	9.0
103A	10/24/50	BF	2	2		1		1.0	2.0
104B	10/24/50	BF	2½			3		3.0	
110A	12/12/50	BF	4½	2	5				18.5
113A	1/16/51	BF	7½	2	2	3		3.0	19.5
208C	1/12/50	EM	4½	2	3	1		1.0	12.5
212A	1/16/51	EM	7½	2	2	2		2.0	9.5

* BF —Breast fed infants.
M—Mutant of a strain of *L. bifidus*.
EM—Infants fed evaporated milk.

ancy is probably explained by the fact that the latter were working with aerobic, unbranched bacilli rather than with microaerophilic, branched organisms. Antigenic studies, together with reviews of the literature on other lactobacilli, were reported by Williams[10] and Orland.[11]

In view of these uncertainties, it was considered desirable to reinvestigate the homogeneity of the group of branched organisms and their unbranched mutants by means of agglutination reactions and cultural requirements.

MATERIALS AND METHODS

Cultures.—Seven strains of *L. bifidus* and two variants of one of them were selected for preparation of antiserums (see table 1). The strains had been isolated from stools of breast-fed infants 5 days to 3 months of age. Strain Jackson, previously described,[1] had been transferred continuously on culture mediums for over 3 years. During this time mucoid colonies were noted for the first time when the culture was plated on December 12, 1949. A typical mucoid colony was picked and maintained as a separate culture designated Jackson M. The characteristics of mucoid variants are to be described elsewhere.[12]

Colonies of unbranched organisms, in contrast with branched organisms, were noted in plates streaked from the Jackson culture on January 12, 1950. When isolated these organisms were found to be aerobic and similar to other unbranched mutants described as *L. parabifidus*.[1] This strain was maintained separately and designated Jackson S. A second strain of *L. parabifidus*, Lockhart S, was employed to produce antiserum, but the agglutinin titer of the antiserum was not satisfactory in spite of repeated inoculations of the animals. This strain was employed, however, for agglutinin adsorption studies as will be described later.

Among the remaining strains listed in table 1, it should be pointed out that strain #212A will not grow in the chemically defined medium of Norris et al,[1] unless breast milk is added as a supplement. The growth requirements of this strain, designated *L. bifidus* var. *Penn.*, will be reported elsewhere.[13]

All of the above strains plus 71 other strains of *L. bifidus* and 4 other strains of *L. parabifidus* were tested for their ability to grow in the simple medium of Hassinen et al.[5] Seventy-nine strains of *L. bifidus* and 6 of *L. parabifidus* were tried in this medium. The sources of the 71 strains of *L. bifidus* were as follows: 25 from stools of breast-fed infants, all designated in the 100 series; 22 from stools of infants fed evaporated milk, assigned numbers in the 200 series; 5 from stools of infants fed S.M.A. (Wyeth Inc.), 300 series; and 19 from vaginal tracts of adults, 400 series. The strains of *L. parabifidus* employed, Lockhart S, Coleman A, Perrish S and #211A(S) are all mutants of strains of *L. bifidus*. The characteristics of strain A were reported by Tomarelli et al.[14,15]

10. Williams, N. B. 1948, J. Infect. Dis. **82**: 31–41.
11. Orland, F. J. 1950, J. Infect. Dis. **86**: 63–80.
12. Norris, R. F., De Sipin, M., Harvey, T. S. and György, P. Manuscript in preparation.
13. György, P., Norris, R. F., Rose, C. S. and Springer, G. F. Manuscript in preparation.
14. Tomarelli, R. M., Norris, R. F., György, P., Hassinen, J. B. and Bernhardt, F. W. 1949, J. Biol. Chem. **181**: 879–888.
15. Tomarelli, R. M., Norris, R. F., Rose, C. S. and György, P. 1950, J. Biol. Chem. **187**: 197–204.

Agglutination tests using antiserums for strains of *L. bifidus* and *L. parabifidus* were made by the macroscopic slide technique or by the conventional test tube method with all of the above strains as well as with the following: *L. bifidus* from infant stools, strains JNX-11, JNX-12 and BLX-15; 9 strains of lactobacilli of human oral origin; and 9 strains from the American Type Culture Collection (ATCC): *L. lactis* #8000; *L. casei* #4946, #4951, and #7969; *L. acidophilus* #4355, #4357, and #9857; and *L. fermenti* #8289 and #9338.

For isolation of *L. bifidus*, fresh stools emulsified in about 10 volumes of sterile 0.85% sodium chloride solution were streaked on solid medium in petri plates. The composition of the modified Teply and Elvehjem[2] medium differed from that described by Tomarelli et al[14] in that lactose, which was substituted for glucose by Norris et al,[1] was reduced from 140 g to 70 g per liter of double strength medium and vitamin B_{12} and pancreatin were omitted. Ascorbic acid was sterilized by Seitz filtration and added aseptically to the autoclaved medium. The final pH was between 6.5 and 6.6. Cultures were incubated in a Brewer[16] jar, under anaerobic conditions, with the addition of 10% carbon dioxide to the atmosphere. Incubation was carried out at 37 C for a period of 40 to 48 hours. Cultures were maintained in liquid medium by making transfers on alternate days.

The simplified medium of Hassinen et al[5] was employed to determine the differences in the complexity of the nutritional requirements of the various strains employed in these studies. Cultures were incubated in the same fashion as described above. Growth or no growth was determined by the presence or absence of significant amounts of titrable acidity in liquid culture medium after 96 hours of incubation.

The production of total, volatile, and lactic acid, as well as the optical rotation of the lactic acid, was determined for the more recently isolated branched strains #103A and #104B, by methods previously reported.[1]

Antigens.–Fresh transplants of the cultures were inoculated into sufficient amounts of liquid medium so that quantities of growth were available for all injections and agglutination tests. Cells were removed from the medium by centrifugation and washed 3 times in sterile 0.85% sodium chloride solution. Suspensions were made in the sterile saline solution so that a turbidity reading of 750, on a Klett-Summerson colorimeter using a filter of 470 mμ, was obtained for a *concentrated vaccine* and a reading of about 280 for a *dilute vaccine*. The suspensions of vaccines were heated at 60 C for 2 hours, tested for sterility, and stored in the refrigerator until used. The suspensions for agglutination tests (see below) were prepared from the same lot of cells in concentrated suspension; phenol was added to a final concentration of 0.2% as a preservative, and the suspensions were stored in the refrigerator. Glass beads were included in the sterile bottles, used for storage of the vaccines and antigens for agglutination tests to facilitate resuspension.

Preparation of antiserums (see table 1).—Young rabbits were bled prior to any injections of vaccine, and the serums were tested for agglutination of the antigen to be injected. Antiserums were not considered satisfactory until the titer of serum agglutinins reached 1:1280. The following injection schedules were employed:

Method A—Intravenous injection of 0.2 ml of dilute vaccine on alternate days until 5 injections were made, followed by preliminary bleeding 1 week after the last injection. The procedure was repeated until a satisfactory titer was found by agglutination test.

Method B—Intravenous injection of 0.5 ml of dilute vaccine on 3 successive days, a rest period of 1 week, then injection of 1 ml on 3 successive days, followed by test bleeding 1 week after the last injection. The 1-ml injections were continued by the latter schedule if the titer of the antiserum was not satisfactory after a single course of injections.

Method C—Intravenous injection of 0.2 ml of concentrated vaccine on alternate days until 5 injections were given, followed by test bleeding 1 week after the last injection. Schedule repeated if necessary.

Method D—One ml of concentrated vaccine was mixed with 4 ml of sterile 5% sodium alginate solution and injected intraperitoneally; then, without removing the needle, 2.5 ml of sterile 1% aqueous calcium chloride solution were introduced through the needle.[17] A period of about 3 weeks was allowed to pass; then 1 ml of dilute vaccine was inoculated intravenously every 4th day until 3 injections had been given. A preliminary bleeding was made 10 days after the last injection; if the titer was unsatisfactory the intravenous injections were repeated.

Animals were bled within 24 to 48 hours after agglutination tests of antiserums, from preliminary bleedings, indicated titers of 1:1280 or

16. Brewer, J. H. 1938–1939, J. Lab. & Clin. Med. **24**: 1190–1192.

17. Slavin, D. 1950, Nature, London, **185**: 115.

TABLE 2.—*Agglutination relationships of eight strains of* L. bifidus *and one strain of* L. parabifidus.

Antiserums	Culture suspensions*								
	Jackson	Jackson M	103A	104B	110A	113A	208C	212A	Jackson S
Jackson	**2560**†	**1280**	**640**	80	40	0	0	0	0
Jackson M	**640**	**1280**	**640**	80	40	0	0	0	0
103A	**1280**	**640**	**2560**	0	0	0	0	0	0
104B	80	80	40	**2560**	**640**	0	0	0	0
110A	0	40	20	**640**	**1280**	0	0	0	0
113A	0	20	0	0	0	**1280**	0	20	0
208C	0	20	0	0	0	0	**2560**	40	40
212A	0	0	0	0	0	0	0	**2560**	0
Jackson S	0	0	0	0	0	0	0	0	**1280**

* Aggutination tests with serums from the same animals prior to preparation of antiserums were negative in lowest dilution employed (1:20).

† Figures represent reciprocals of highest antiserum dilutions in which at least 3+ agglutination occurred. All controls were negative.

higher. Antiserums were treated with phenol to a final concentration of 0.2% and stored in the refrigerator.

Agglutination tests.—A macroscopic slide agglutination test was used for preliminary studies of relationships between various cultures before use of the test tube agglutination method. Antigen for slide agglutination tests was prepared from the concentrated bacterial suspension (See *Antigens*) by filtering the suspension through cotton in a pipette or glass tubing while applying negative pressure. If spontaneous agglutination occurred, filtration was repeated until an even suspension was obtained. Antiserums were employed undiluted or diluted 1:10 in 0.85% sodium chloride solution. The antigens and antiserums were mixed and observed in the fashion described by Williams.[10] Negative slide agglutination tests were not checked by the test tube technique.

All serums employed in the test tube technique were diluted by the doubling dilution method with 0.85% sodium chloride solution. The final dilutions of serums were between 1:20 and 1:5120. Antigen was prepared for these tests by filtering the bacterial suspension from the concentrated suspension, as described above, then diluting with 0.85% sodium chloride solution until a turbidity of 145 on the colorimeter was obtained. The serum-antigen mixtures were incubated for 2 hours in a water bath at 37 C, then stored in the refrigerator overnight. Readings were made the next day against a dark background by indirect lighting. Controls were checked prior to reading, and if the cells did not resuspend evenly, the test was discarded. Results were recorded as 4+ for complete, 3+ as incomplete and 2+ as partial agglutination. The homologous titer was arbitrarily set as the highest dilution, exhibiting 3+ or stronger agglutination.

Adsorption technique.—All antiserums were diluted 1:10 prior to mixture with washed cells sedimented by centrifugation. A 10-ml portion of the diluted antiserum was mixed with 0.5 ml of the packed cells in a sterile test tube, plugged with a rubber stopper, and agitated by hand to bring about an even suspension. The test tube was attached to a mechanical mixing device and the apparatus placed in an incubator at 37 C for 2 hours. The bacterial cells were then removed by centrifugation and the above procedure was repeated 3 times, using fresh cells on each occasion. An agglutination test was then performed. Most agglutinins were removed by this treatment, but in some instances one more adsorption was necessary.

RESULTS

The data from agglutination tests with both homologous and heterologous organisms, shown in table 2, indicate the antigenic relationships between 8 strains of *L. bifidus* and 1 of *L. parabifidus*. Cross reactions which are considered significant are shown in boldface figures. Reactions not considered significant are shown in ordinary type. One group relationship among the branched strains exists between Jackson, Jackson M, and #103A, and another distinct group is formed by strains #104B and #110A. Strains #113A, #208C and #212A appear to be antigenically distinct from each other and from the two groups just described. Strain Jackson S, although derived from Jackson, is distinct serologically from all of the branched strains.

The results of selected agglutinin adsorption tests are presented in table 3. All of the antiserums were adsorbed with homologous organisms, but the results are not shown since adsorption

was complete in each instance, and all homologous agglutination tests were negative in the lowest dilutions of antiserums tested (1:20). All antiserums prepared with Jackson, Jackson M, #103A, #104B and #110A were adsorbed with cells from each heterologous strain, but only the tests that are pertinent to the distinction of antigenic components among these strains are shown in table 3. In the case of antiserums #113A, #208C, #212A, and Jackson S, agglutinin adsorptions were not done with all of the heterologous antigens. In no instance, however, was the antiserum titer for the homologous organisms reduced by adsorption with heterologous strains. A comparison of the data in tables 2 and 3 indicates that the antigenic differences, revealed by agglutination tests with unadsorbed antiserums, are confirmed by agglutinin adsorption tests. The following designations of surface antigens have been selected and will be explained in the *Discussion*.

Component IIa·1.—The data in table 2 indicate that strains Jackson, Jackson M and #103A are closely related and distinct from other strains. Adsorption of Jackson antiserum with Jackson M or #103A organisms removes the agglutinins for all strains (table 3). The results are similar after mirror adsorptions using Jackson, Jackson M, or #103A antiserum. Adsorption of Jackson, Jackson M or #103A antiserum with #110A or #104B organisms does not alter the homologous titers or the titers for other strains in this group. Adsorption of Jackson antiserum with Jackson S organisms likewise does not significantly reduce the titer. Jackson S is also serologically distinct from Jackson M and #103A. The designation IIa·1 has been assigned to the antigenic component in the strains Jackson, Jackson M and #103A.

Components IIa·2, 3, 4, 5.—Among the organisms tested, it is seen that strains #104B and #110A constitute a second group and that strains #113A, #208C and #212A are individually distinct from any other organism. The designations above have been assigned

TABLE 3.—*Agglutinin adsorption tests.*

Antiserums	Adsorbed with antigen	Culture suspensions									Antibodies in adsorbed antiserums*
		Jackson	Jackson M	103A	104B	110A	113A	208C	212A	Jackson S	
Jackson	Unadsorbed	2560†	1280	640	80	40	0	0	0	0	
	Jackson M	0	0	0	0	0	0	0	0	0	
	103A	0	0	0	0	0	0	0	0	0	
	110A	2560	1280	640	0	0	0	0	0	0	
	Jackson S	1280	640	640	0	0	0	0	0	0	IIa·1
103A	Unadsorbed	1280	640	2560	0	0	0	0	0	0	
	Jackson	0	0	0	0	0	0	0	0	0	
	104B	640	640	1280	0	0	0	0	0	0	IIa·1
110A	Unadsorbed	0	40	20	640	1280	0	0	0	0	
	104B	0	0	0	0	0	0	0	0	0	
	Jackson	0	0	0	640	640	0	0	0	0	IIa·2
	103A	0	0	0	640	640	0	0	0	0	
113A	Unadsorbed	0	20	0	0	0	1280	0	20	0	
	212A	0	0	0	0	0	1280	0	0	0	IIa·3
208C	Unadsorbed	0	20	0	0	0	0	2560	40	40	
	Jackson M	0	0	0	0	0	0	2560	0	0	IIa·4
212A	Unadsorbed	0	0	0	0	0	0	0	2560	0	
	104B	0	0	0	0	0	0	0	2560	0	IIa·5
Jackson S	Unadsorbed	0	0	0	0	0	0	0	0	1280	
	Lockhart S	0	0	0	0	0	0	0	0	0	
	Jackson	0	0	0	0	0	0	0	0	1280	IIb·1

* Each antiserum gave a negative agglutination reaction after adsorption with homologous organisms.
† Figures represent reciprocals of highest dilution in which 3+ agglutination occurred.

TABLE 4.—*Agglutination reactions and cultural tests.*

Strains	Source*	Growth in simplified medium†	Antiserums					
			Jackson Jackson M‡	#104B #110A‡	#113A	#208C	#212A	Jackson S
			Components					
			IIa·1	IIa·2	IIa·3	IIa·4	IIa·5	IIb·1
Jackson	BF	+	+	0	0	0	0	0
Jackson M	M	+	+	0	0	0	0	0
Lockhart	BF	+	+	0	0	0	0	0
Birch	BF	+	+	0	0	0	0	0
Perrish	BF	+	+	0	0	0	0	0
Timberlaine	BF	+	+	0	0	0	0	0
103A	BF	+	+	0	0	0	0	0
104B	BF	0	0	+	0	0	0	0
107A	BF	+	0	+	0	0	0	0
108A	BF	+	+	0	0	0	0	0
105C	BF	0	0	0	0	+	0	0
110A	BF	0	0	+	0	0	0	0
113A	BF	0	0	0	+	0	0	0
116C	BF	0	0	0	0	+	0	0
117C	BF	0	0	0	0	+	0	0
203B	EM	0	0	+	0	0	0	0
204B	EM	0	0	+	0	0	0	0
208C	EM	0	0	0	0	+	0	0
212A	EM	0	0	0	0	0	+	0
214B	EM	0	0	0	0	0	0	+
217B	EM	0	+	0	0	0	0	0
410	VT	0	0	0	0	+	0	0
419	VT	0	0	0	0	+	0	0
422	VT	0	0	+	0	0	0	0
JNX-11	BF		0	+	0	+	0	0
JNX-12	BF		0	+	0	0	0	0
BLX-15	BF		0	+	0	+	0	0
Jackson S	M	0	0	0	0	0	0	+
Lockhart S	M	0	0	0	0	0	0	+
Perrish S	M	0	0	0	0	0	0	+
Coleman A	M	0	0	0	0	0	0	+
Strain A	M	0	0	0	0	0	0	+
211A (S)	M	0	0	0	0	0	0	+
#8000 *L. lactis*	ATCC		0	0	0	0	0	+

* BF —Breast fed infants.
EM —Infants fed evaporated milk.
VT —Vaginal tract of pregnant women.
M —Mutant of a strain of *L. bifidus*.
ATCC—American Type Culture Collection.
† —Hassinen et al.[5]
‡ —Tests included both antiserums. In no case were the tests positive in one and negative in the other.

to these four groups, respectively.

Component IIb·1.—The strains of *L. parabifidus*, Jackson S and Lockhart S are related genetically to the respective parent strains of *L. bifidus* (Jackson and Lockhart) but differ from them in morphological and biochemical characteristics.[1] Therefore, the Roman numeral II plus the letter b are used to distinguish strains of *L. parabifidus* from *L. bifidus*. Neither Jackson S nor Lockhart S organisms are agglutinated in any of the II·a antiserums. Even though a satisfactory antiserum could not be prepared with strain Lockhart S, an antigenic relationship with strain Jackson S is indicated by agglutination of Lockhart S organisms in Jackson S antiserum, and adsorption of all agglutinins from this antiserum. The designation IIb·1 is employed to indicate the antigenic component apparently held in common by these two strains of *L. parabifidus*.

A number of strains of *L. bifidus*, *L. parabifidus* and other kinds of lactobacilli (see *Cultures*) were tested for agglutination in antiserums representing the different components. The results of these tests are presented in table 4 and show that 27 of 82 strains of *L. bifidus* were agglutinated by one or more antiserums. Nine strains were agglutinated in IIa·1, 9 in IIa·2, 8 in IIa·4 antiserum. None of the strains, other than the homologous organisms, were agglutinated in IIa·3 or IIa·5 antiserum. Strains JNX-11 and BLX-15 (freshly

isolated from the stools of new born partially breast-fed infants) are the only organisms tested thus far that were agglutinated by more than one antiserum. Reactions occurred in IIa·2 and IIa·4 antiserums. Strain #214B is the only *L. bifidus* we have tested that agglutinated in the IIb·1 antiserum. This strain was studied repeatedly and just recently was found to have changed so that it demonstrates the characteristics of a strain of *L. parabifidus*.

Six strains of *L. parabifidus* were agglutinated in the IIb·1 antiserum. Strain #211A(S) was a typical branched strain when examined on January 21, 1951, but was not agglutinated by any of the IIa antiserums. Examination of the culture in July, 1951 revealed that the organisms were *unbranched bacilli* and agglutinated only in IIb·1 antiserum.

L. lactis #8000 was agglutinated in the IIb·1 antiserum. None of the other ATCC cultures or of the 9 human oral strains of lactobacilli was agglutinated in any of the antiserums.

Many of the strains of *L. bifidus* and *L. parabifidus* were tested for agglutination in monospecific antiserums for antigenic components A through I found in certain other kinds of lactobacilli.[10,11] None of the strains was agglutinated by any antiserum.

Nearly all of the strains of *L. bifidus* and *L. parabifidus* were tested for their ability to grow in the simple liquid medium of Hassinen et al.[5] There were 9 strains that demonstrated this capacity, as indicated in table 4. Strains Lockhart, Perrish, Timberlaine, and Birch were the four strains used by Hassinen et al.[5] Thus, their observations are confirmed. On the other hand, no recently isolated strains have been found capable of growing in this medium. All of the strains which grew in the liquid medium, except strain #107A, are agglutinated by the IIa·1 antiserum.

TABLE 5.—*Acid production by strains of* L. bifidus.

Strains	Total acid	Volatile acid	Lactic acid	Configuration of lactic acid
	Ml of 0.1 *N* acid per 10 ml medium			
103A	21.6	12.5	10.6	L(+)
104B	12.8	8.3	6.6	L(+)

The results of a study of the production of total acids, volatile acid, and lactic acid by strains #103A and #104B are presented in table 5. The amount of volatile acid, which is almost entirely acetic acid, exceeds the amount of lactic acid. The latter in all cases is *dextro*-rotatory. These observations are in agreement with results obtained for strains isolated at an earlier date.[1]

DISCUSSION

As can be seen from the schedule of inoculations, the antigenicity of the different strains varied markedly. In some instances antiserums were produced in rabbits with great difficulty, in others with little or no difficulty. We have not found a single injection procedure which is satisfactory for all strains regardless of morphology. Repeated injections of bacteria as antigens over long time periods usually lead to antiserums that cross react with more heterologous antigens than antiserums prepared over short periods of time. This is due to an increase in the concentration of antibodies against lesser antigens contained within the organisms.[18] In spite of the long period of time required for the production of antiserums with titers of 1:1280 or higher, there was no increase in cross reactions. Blaurock[6,7] and Boventer[8] also reported production of antiserums having high agglutinin titers for

18. Arkwright, J. A. 1931, A system of bacteriology in relation to medicine. London, Privy Council, Med. Res. Council, **6**: 406–408.

the homologous organisms. However, Boventer[8] did not seem to have success with single strains and finally resorted to the use of polyvalent vaccines. These workers make no reference to branched or unbranched strains.

The antigenic analysis was performed even though only a few of the strains demonstrated cross reactions. Reciprocal adsorptions, in addition to the heterologous agglutination tests, indicate that five antigenic components, IIa·1, 2, 3, 4, 5, were identified among the strains of *L. bifidus* and a single antigenic component, IIb·1, was associated with the strains of *L. parabifidus* studied thus far.

Studies of antigenic components of lactobacilli have been limited largely to "surface" components which are detectable by agglutination tests.[19] Since only 27 of the 82 branched strains were agglutinated in antiserums known to contain particular agglutinins, it is apparent that other surface antigens among strains of *L. bifidus* probably exist. In the case of those strains which were agglutinated by the various antiserums, the number is about equally divided among IIa·1, 2 and 4.

Inasmuch as the Jackson S strain is believed to be a mutant of the Jackson strain, the loss of antigenic relationship with all branched strains tested, except for #214B, is especially interesting. Strain #214B, when isolated late in 1950, had all of the characteristics of other strains of *L. bifidus*, and was carried in stock by making transfers in broth on alternate days. Agglutination tests with a suspension of the organisms in February, 1951 were negative for all antiserums. The tests were repeated in July, 1951 and agglutination occurred to a high titer in the IIb·1 antiserum only, even though culturally it was a microaerophilic, branched bacillus. In September the strain was lyophilized. It was recultured in March, 1952 and carried in stock broth again as described above. It still exhibited all of the characteristics found originally. In August and early September platings revealed a mixture of colonies; some contained organisms with branching, others organisms with no branching. By the middle of September the plates contained only colonies of unbranched organisms. Agglutination tests with a suspension of the unbranched organisms show that agglutination still occurs only in the IIb·1 antiserum. It is apparent that this strain, at the time of the second agglutination tests, contained a mixture of unbranched mutants for which there was an antiserum, and branched organisms for which there was no antiserum. Since this strain subsequently changed to an aerobic, unbranched bacillus the early agglutination in antiserum from another unbranched bacillus seems to have indicated, in advance, that a transformation was imminent. It appears that a similar change occurred in the strain labelled #211A(S), for it was a microaerophilic, branched bacillus when first isolated, but now is an unbranched bacillus and agglutinated in IIb·1 antiserum. Agglutination of *L. lactis* #8000 in IIb·1 antiserum suggests that there may be antigenic cross reactions between homofermentative and heterofermentative organisms.

Although only three strains of *L. acidophilus* (ATCC) were tested in all of the antiserums, the absence of any agglutination lends support to the view that these organisms differ in so many characteristics as to warrant separate species identification.

The designations for the antigenic

19. A symposium: the nature of the bacterial surface, 1949, The Society for General Microbiology, Oxford, Blackwell Scientific Publications, pp. 106–122.

components are made from a plan designed to distinguish between kinds of lactobacilli, as well as different types of antigenic components, as follows:

A proposal for labelling antigenic components among members of the genus Lactobacillus

I. Homofermentative lactobacilli—produce primarily lactic acid; morphologically unbranched bacilli; aerobic or microaerophilic.
   I—. *A dash or minus sign* following the Roman numeral signifies no subdivision of these microorganisms. If grounds for morphologic or cultural subdivisions are found by future research, lower case letters are suggested (see subdivisions of heterofermentative lactobacilli).
      1. *Arabic numbers* (to follow I—)—a different number for each separate "surface" (not capsular) component. The arabic numbers 1 to 9 would replace the capital letters A to I, which have already been assigned.[10,11]
         a. *Lower case letters* (to follow Arabic number)—a different letter for each separate protein antigen.[20]

II. Heterofermentative lactobacilli—produce by products in addition to lactic acid, e.g., carbon dioxide, alcohol, and acetic acid.
   IIa. Produce volatile acids *and* lactic acid; no gas; branched bacilli; microaerophilic.
      1. *Arabic numbers* (to follow IIa)—a different number for each separate surface (not capsular) component.
   IIb. Produce less lactic acid than homofermenters; variable amounts of gas. Unbranched bacilli; aerobic and microaerophilic.
      1. *Arabic numbers* (to follow IIb)—a different number for each separate surface (not capsular) component.

The use of the plan may be visualized if a hypothetic formula is analyzed, e.g., IIb·1, 2., IIa·4., I—·1. The designation IIb indicates the kind of organism tested, and the arabic numbers 1 and 2 refer to surface components within that group of organisms. The IIa indicates a cross with a second group of lactobacilli and the number 4 refers to a relationship with a specific antigenic component in this group. The I—·1 refers to a relationship with a third group and the specific component. No attempt has been made to consider species names along with these designations, since strains of lactobacilli with identical names may possess different antigenic components.

Although the homofermentative and heterofermentative groups of lactobacilli described in Bergey's Manual[4] have been used as the basis for the above differentiation of kinds of lactobacilli, it will be noted that the subgroups under the heterofermentative group are separated, in addition to other characteristics, on the basis of cellular morphology and production of varying amounts of gas. *L. bifidus* would be placed in the first subgroup and *L. parabifidus* in the second subgroup. Such an arrangement is contrary to Bergey's Manual,[4] since *L. bifidus* is listed with homofermentative lactobacilli and *L. parabifidus* is described along with *L. bifidus*. According to this plan, the other types of heterofermentative lactobacilli, i.e., *L. brevis*, *L. buchneri*, etc., also would be placed in the second subgroup.

Allowance has been made for sub-

20. Jackson, S. W. and Williams, N. B. Unpublished work.

division of the homofermentative lactobacilli, extension of the subdivision of the heterofermentative group, and addition of other types of antigenic components that may be found by future research. It will be noted that no provision is made for a cellular carbohydrate component. Harrison, Zidek and Hemmens[21] found that strains of homofermentative lactobacilli contained a serologically reactive carbohydrate. They reported two groups: one contained four or more serologic types; the other comprised serologically heterogeneous strains. The studies of Williams[10] indicated that the carbohydrate precipitating antibody and the agglutinating antibody of some of the strains of lactobacilli used by Harrison et al.[21] are identical. It appears to be a duplication, therefore, to assign a separate symbol to carbohydrate extracts of this nature. Preliminary studies in this laboratory[20] indicate that a serologically reactive protein, not related to the surface components, may be obtained from homofermentative lactobacilli. Therefore, a tentative distinguishing symbol for such antigens has been included in this group. Certain strains of *L. bifidus* have been noted to produce extracellular polysaccharides[12,22] If these substances are employed to differentiate strains, a distinctive symbol can be assigned. It is proposed that arabic numbers and lower case letters be employed alternately, and in sequence, as different substances are discovered. The proposal for designation of antigenic components among organisms in the genus *Lactobacillus* has thus been utilized to designate the five distinct components among the strains of *L. bifidus* studied as IIa·1, 2, 3, 4, 5 and the single component among the strains of *L. parabifidus* studied as IIb·1.

The tests for capacity to grow in the simplified medium of Hassinen et al[5] indicate that growth of few strains of *L. bifidus* and no strains of *L. parabifidus* is supported by this medium. On the other hand, the data suggest a relationship between growth in the simplified medium and presence of a particular antigenic component. Only one strain out of the nine found to grow in the simplified medium was not agglutinated in the IIa·1 antiserum. Studies of the antigenic relationships of a strain before and after development of the capacity to grow in the simplified medium would be of particular interest. This is especially true in view of the report of Harrison[23] that antigenic specificity of certain strains of lactobacilli changed at the time of alteration in fermentative capacity.

The agglutination tests of branched and unbranched strains indicate differences between these strains which are demonstrable on the basis of morphology and biochemical activity. Thus, it seems to the authors that the data support the application of the name *L. bifidus* to the branched strains and *L. parabifidus* to the aerobic unbranched derivatives of the branched strains. There is still the possibility that more relationships may be demonstrated among the lactobacilli when more complete antigenic analyses are available.

SUMMARY

1. The agglutination and agglutinin adsorption studies of 8 strains of *Lactobacillus bifidus* led to the recognition of 5 distinct antigenic components, designated IIa·1, 2, 3, 4, 5. A total of 27 out

21. Harrison, R. W., Zidek, Z. C. and Hemmens, E. S. 1939, J. Infect. Dis. **65**: 255–262.
22. Malyoth, G. and Bauer, A. 1950, Ztschr. f. Kinderh. **68**: 358–67; 1951. Ztschr. f. Biol. **104**: 404–408.
23. Harrison, R. W. 1942, J. Infect. Dis. **70**: 77–97.

of 82 strains of *Lactobacillus bifidus* was agglutinated in one or more of the antiserums, indicating that all antigenic components have not been recognized.

2. A satisfactory antiserum could be prepared with only one of two strains of *L. parabifidus*. Agglutination and agglutinin adsorption tests between these strains and strains of *L. bifidus* led to the recognition of an antigenic component designated IIb·1. All strains of *L. parabifidus* tested in this antiserum were agglutinated.

3. All branched and unbranched strains grow in the medium employed in this work. No *freshly* isolated strains of *L. bifidus* and none of the strains of *L. parabifidus* were capable of growing in a simplified medium. All strains of *L. bifidus* capable of growing in the simplified medium, except one, were agglutinated in the IIa·1 antiserums.

4. A plan, based on the data presented in this paper and the work of others, is proposed for assigning symbols to antigenic components of homofermentative and heterofermentative lactobacilli.

THE DEVELOPMENT OF ARSENIC RESISTANCE IN *TRYPANOSOMA GAMBIENSE* AND ITS INFLUENCE ON PARASITEMIA

ELEANOR JOHNSON TOBIE AND THEODOR VON BRAND

From the Federal Security Agency, Public Health Service, National Institutes of Health, Laboratory of Tropical Diseases of the National Microbiological Institute, Bethesda, Maryland

The occurrence of arsenic-resistant strains of trypanosomes is very troublesome in practical chemotherapy of African trypanosomiasis. The phenomenon has been recognized for a long time but very little has been known about its mechanism, and as far as we are aware no one has observed a change in the pattern of infection concomitant with the development of resistance. In order to study the metabolic changes in arsenic-resistant *Trypanosoma gambiense*, which will be reported elsewhere (von Brand et al, in press), it was necessary for us to develop a strain highly resistant to an arsenical. We agree with Yorke et al (1931) that the term "arsenic resistance" when applied to a strain made resistant to an organic arsenical is somewhat misleading, since the resistance is almost exclusively directed against the organic part of the drug molecule. However, in conformity with general usage we shall continue to use the term.

In the present study the development of resistance to reduced tryparsamide in *T. gambiense*, and the changes in the pattern of infection in white rats, white mice, and guinea pigs which appeared after the strain had reached a high degree of resistance, will be discussed.

Received for publication September 4, 1952.

We are indebted to Nathan Mantel for statistical advice, to Benjamin Mehlman for preparing the required drug solutions, and to Kenneth B. Gilpin, James L. Hancock, and Andrew H. Kirkpatrick for technical assistance.

MATERIALS AND METHODS

The Wellcome TS strain of *T. gambiense* was used as the normal strain in this study. It was received in February, 1947 through the courtesy of Dr. C. A. Hoare, from the Wellcome Laboratories of Tropical Medicine, London, where it had been maintained in mice since 1934. Its beginning as a laboratory strain followed isolation from a patient at the Hospital for Tropical Diseases, London, in November, 1921. In 1947 mice infected with this strain survived for 3 to 4 days. In our laboratory our stock strain has been maintained principally in guinea pigs, which survive 2 to 4 weeks, depending on the number of parasites given as inoculum. The drug-resistant strain was produced from the normal strain described above, except that a white rat had been infected to serve as the donor host. We chose to use rats as donor and test hosts since the disease produced by *T. gambiense* progresses more rapidly in rats and because a single rat would yield infected blood in amounts adequate for the control experiment and the exposure to various drug concentrations.

Resistance was developed to reduced tryparsamide, the same compound used by Yorke, Murgatroyd, and Hawking (1931). The parasites were exposed in vitro to successively higher concentrations of the drug essentially as described by the above authors for producing resistance in *Trypanosoma rhodesiense*. The details of our procedure were as follows: An infected rat with a high parasitemia was anesthesized, as much blood as possible was drawn from its heart, and the blood defibrinated and mixed with dextrose to bring the glucose content to about 400 mg percent. Ringer-serum solution* was added to this defibrinated blood to make a volume of 10 ml. This was centrifuged at low speed, 1000 to 1200 r.p.m. (relative centrifugal force 170 to 250) for 3 minutes to remove the red blood cells,

* Fifty percent rabbit serum and fifty percent Ringer solution: sodium chloride, 0.9 g; potassium chloride, 0.025 g; calcium chloride, 0.02 g; glucose, 0.2 g; distilled water, 100 ml.

and the supernatant containing the trypanosomes pipetted to another tube. Usually it was necessary to centrifuge a second time at low speed to remove the remaining red blood cells. The supernatant was then centrifuged at high speed, 3300 to 3500 r.p.m. (relative centrifugal force 1750 to 2090) for 5 minutes to spin down the trypanosomes, and 0.5 ml Ringer-serum was added to the trypanosome sediment. After thorough mixing, 0.1 ml of the suspension was added to each of 5 tubes, 4 containing 0.8 ml Ringer-serum plus 0.1 ml Ringer solution containing a specified drug concentration and one, a control, identically prepared except that no drug was added to the Ringer solution. After incubation at 37 C for 1 hour, during which time the tubes were shaken about every 15 minutes, the trypanosomes were centrifuged at high speed and washed twice in 3 ml Ringer-serum solution. After the final washing 1 ml Ringer-serum was mixed with the trypanosome sediment in each tube and, where survivors were found microscopically, fresh test rats were inoculated intraperitoneally with the suspension. The next donor was selected from test rats inoculated with trypanosomes which had been exposed to the highest drug concentration at which survival and infectivity were retained.

Studies on the pattern of infection were begun about 5 months after the strain had become about 600 times more resistant to reduced tryparsamide than the normal strain. During the interval the strain had been maintained by serial transfer through rats. Metabolic studies carried out at approximately the time the infectivity studies were begun showed that this type of maintenance had no influence on the resistance level. This is in agreement with Fulton and Yorke (1941), who observed that arsenic resistance once established is an extremely stable property of trypanosomes.

The 2 strains were compared in 3 different host animals, white rats, white mice, and guinea pigs. In each experiment animals were inoculated intraperitoneally with definite numbers of trypanosomes per 100 g body weight. The parasite numbers were estimated by hemocytometer counts, and proper dilutions were made with Ringer-serum solution to allow a 0.2 ml suspension to be inoculated. In each experiment animals were all of the same sex and of approximately the same weight. Rats averaged 200 g, mice 15 to 18 g, and guinea pigs 200 to 220 g. The degree of parasitemia, in all experiments except one where hemocytometer counts were made, was determined by microscopic examination of fresh drops of blood from the tail of the rats and mice and from the ear of the guinea pigs. Records were made according to the following scheme: p = trypanosomes difficult to find (1, 2, or 3 in a drop); + = trypanosomes easy to find (up to 3 to 4 per 4 mm objective field); ++ = trypanosomes more numerous (could be counted in a thin drop under the 4 mm lens); +++ = trypanosomes numerous (could not be counted under the 4 mm lens); ++++ = trypanosomes extremely heavy (usually limited to extreme numbers just prior to death). Animals were checked twice daily for survival.

RESULTS AND DISCUSSION

Development of the arsenic-resistant strain

The manner in which resistance to reduced tryparsamide developed in our strain of *T. gambiense* is shown in figure 1, which also shows the data given by Yorke, Murgatroyd and Hawking (1931) concerning the development of resistance by *T. rhodesiense* to the same compound. It is evident that during the first 19 exposures *T. gambiense* showed no sign of increasing resistance. There were, however, minor variations in resistance, and it should be noted that at the 16th exposure the resistance was definitely lower than either before or after that point.

During the first 16 exposures horse serum (Difco Bacto horse serum—normal without preservation) was used in the Ringer-serum medium. At about this time physiological studies, using the strain maintained in rats at the 13th exposure level, showed that horse serum was not entirely satisfactory and that the trypanosomes survived better in rabbit serum. Beginning with the 17th exposure rabbit serum was used in the medium, and following the 19th exposure the resistance began to develop rapidly and reached a degree of resistance high enough for our use following the 25th exposure. It is possible that the trypanosomes at the 16th exposure were damaged by the horse serum in the higher drug concentrations rather than by the drug itself or that some other undetected factor, such as an unfavorable pH, occurred.

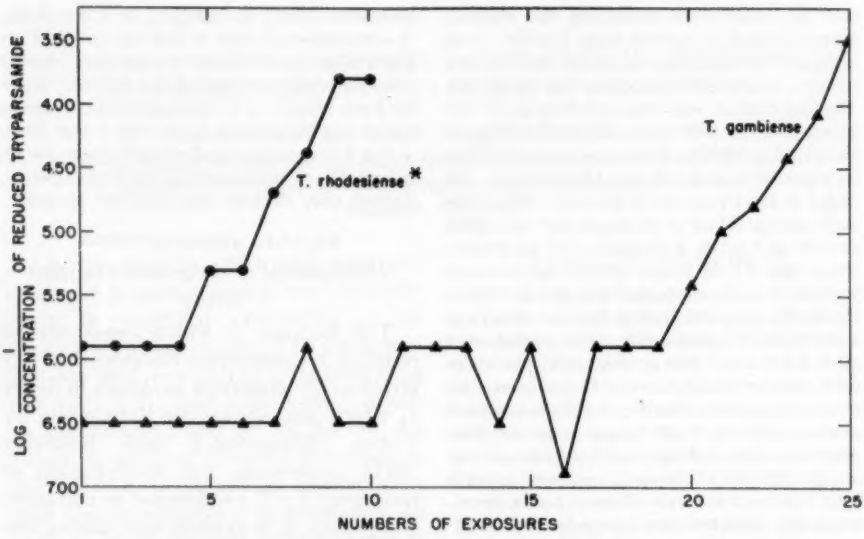


FIG. 1.—Development of resistance to reduced tryparsamide in *T. rhodesiense* and *T. gambiense;* drug concentration at which trypanosomes survived exposures and were capable of infecting test rats.
* Data of Yorke et al. (1931).

Figure 1 shows clearly that the course of development of resistance was identical in *T. rhodesiense* and in *T. gambiense*, even though at the start the former survived a higher drug concentration, developed resistance more rapidly, and finally did not tolerate quite as high a concentration of drug as *T. gambiense*. The latter initially survived a concentration of 1:3,200,000 and after the 25th exposure, 1:3,125. However, this does not indicate a 1000-fold increase in resistance, since in the early exposures the next higher concentration tested was 1:800,000, which proved lethal. It is probable that the trypanosomes would have survived some concentration between the two. Metabolic experiments indicate about a 600-fold increase in resistance.

The literature on drug resistance in trypanosomes, summarized by von Brand (1951), indicates that most investigators favor the hypothesis that grades of resistance, so high they cannot be explained by a simple selection of the most resistant individuals of a mixed population, are due to mutation. We agree that some fundamental change has to be postulated, but wish to point out that the sudden, stepwise development of resistance is difficult to understand on the basis of a single mutation, unless some auxilliary hypothesis is invoked. Whether successive mutations, plasmatic inheritance according to Sonneborn (1949), or long lasting modifications according to the interpretations of Jollos (1921) are involved cannot be decided at the present time. As pointed out by von Brand (1951), these features are difficult, if not impossible, to analyze in organisms that do not have sexual reproduction.

The course of infection in rats, mice, and guinea pigs

The average number of days mice, rats, and guinea pigs survived infection with the normal and the drug-resistant

strains of *T. gambiense* and the types of infection resulting in these animals are summarized in table 1. It is shown that in the three host species studied the average survival of the animals infected with the arsenic-resistant parasites was significantly longer than that of those infected with an equal number of non-resistant trypanosomes. The prepatent period (table 1) was slightly longer in each instance, with the exception of the rats inoculated with 1,000,000 trypanosomes, in which case it was exactly the same. It is obvious, then, that the difference in the pattern of infection is due to the difference in the length of the patent period. Two types of infections were recognized in the host animals: (1) a *progressive* type, in which the parasites steadily increased in numbers in the bloodstream to a crucial point with death the result, and (2) a *relapsing* type, in which the parasites increased, then dropped noticeably in number only to increase again until death of the host resulted.

With both the normal and arsenic-resistant strain the infection in mice followed solely the progressive pattern, while in guinea pigs the relapsing type was the exclusive pattern. However, in a number of guinea pigs several relapses occurred and between these relapses minor but noticeable fluctuations in parasitemia were evident. This agrees with the known infection pattern of practically all trypanosomes of the *brucei* and the *evansi* groups (Reichenow, 1929). However, a different picture was presented by rats. All 45 infected with the normal strain (table 1 and table 2), regardless of size of inoculum, showed the progressive type of infection, but of 49 rats infected with the resistant strain (table 1 and table 2), only 14 exhibited the progressive pattern while 35 showed the relapsing pattern. Only one relapse was evident; so the first relapse strain proved fatal.

Results of a study on the progress of parasitemia in 10 rats, 5 infected with the normal strain and 5 with the drug-resistant strain, are presented in table 3. Counts given show lower numbers of parasites in the bloodstream of animals infected with the drug-resistant strain

TABLE 1.—*Comparison of a normal and an arsenic-resistant strain of* T. gambiense *in various vertebrate hosts.*

Host	Trypanosomes inoculated per 100 g body weight	Strain	Number of animals	Average survival in days	Average prepatent period in days	Average patent period in days	Type of infection	
							Progressive	Relapsing
Mice	50,000	Resistant	25	5.4±0.10‡ (4.5–6.5)†	1.0±0.04‡ (0.5–1.5)†	4.4±0.12‡ (3.5–6.0)†	25	0
		Normal	25	4.1±0.03 (4.0–4.5)	0.6±0.04 (0.5–1.5)	3.5±0.05 (3.0–4.0)	25	0
Rats	1,000,000	Resistant	25	7.9±0.30 (3.5–9.5)	0.5±0.00 (0.5)	7.4±0.30 (3.0–9.0)	7	18
		Normal	25	3.6±0.06 (3.5–4.5)	0.5±0.00 (0.5)	3.1±0.06 (3.0–4.0)	25	0
	50,000	Resistant	5*	9.3±0.30 (8.5–10.5)	1.7±0.6 (0.5–3.5)	7.6±0.20 (7.0–8.0)	0	5
		Normal	5*	4.9±0.30 (4.0–5.5)	0.9±0.2 (0.5–1.5)	4.0±0.20 (3.5–4.5)	5	0
Guinea pigs	50,000	Resistant	10	46.9±3.80 (26.0–63.5)	2.4±0.30 (1.5–4.5)	44.5±3.50 (24.5–59.0)	0	10
		Normal	10	20.5±2.80 (9.5–37.5)	1.9±0.40 (0.5–4.5)	18.6±2.60 (9.0–35.0)	0	10

* Identical with rats listed in table 2 inoculated with 100,000 trypanosomes (50,000/100 g body weight).
† Range in days.
‡ Average ± standard error.

TABLE 2.—*The effect of the number of parasites inoculated on the pattern of infection in rats infected with a normal and an arsenic-resistant strain of* T. gambiense.

Number of trypanosomes inoculated	Strain	Number of rats		Average survival in days after			Type of infection	
		Inoculated	Infected	Inoculation	Appearance in blood	Relapse	Progressive	Relapsing
100	Resistant	5	1	9.0	3.5		1	0
	Normal	5	2	7.7±0.7† (7.5–8.0)*	3.7±0.7† (3.0–4.5)*		2	0
1,000	Resistant	5	3	13.5±1.7 (11.5–16.5)	9.0±1.0 (8.0–11.0)	3.0	2	1
	Normal	5	3	9.5±2.5 (7.0–14.5)	4.6±0.2 (4.5–5.0)		3	0
10,000	Resistant	5	5	11.3±0.8 (9.5±13.5)	7.4±0.5 (6.0–8.5)	3.0±0.4 (2.5–4.0)	1	4
	Normal	5	5	5.9±0.2 (5.5–6.5)	4.2±0.2 (4.0–5.0)		5	0
100,000	Resistant	5	5	9.3±0.4 (8.5–10.5)	7.6±0.2 (7.0–8.0)	3.0±0.4 (2.0–4.0)	0	5
	Normal	5	5	4.9±0.3 (4.0–5.5)	4.0±0.2 (3.5–4.5)		5	0
1,000,000	Resistant	5	5	6.2±1.0 (4.0–9.0)	5.7±1.0 (3.5–8.5)	3.3±0.3 (3.0–3.5)	3	2
	Normal	5	5	4.0±0.2 (3.5–4.5)	3.5±0.2 (3.0–4.0)		5	0
10,000,000	Resistant	5	5	9.2±0.2 (8.5–9.5)	8.8±0.2 (8.0–9.0)	3.7±0.2 (3.0–4.0)	0	5

* Range in days.
† Average ± standard error.

than in those infected with the normal strain on days when a comparison can be made. Even after the trypanolytic crisis the numbers did not increase as rapidly in the drug-resistant strain as in the normal strain. This indicates that the rate of reproduction of the arsenic-resistant trypanosome may be slower than that of the nonresistant trypanosome, but, of course, does not prove that it is the factor causing the lower numbers.

The fact that the majority of rats infected with the resistant strain showed the relapsing type of infection indicates clearly that rather powerful immune

TABLE 3.—*Parasitemia in rats infected with a normal and an arsenic-resistant strain of* T. gambiense, *each rat inoculated with 100,000 trypanosomes. Daily counts given in millions per ml.*

Rats	Days											Day of death
	1	2	3	4	5	6	7	8	9	10	11	
					Normal strain							
1	p	p	5.0	150.0	1050.0 Died							5
2		p	4.0	160.0	1210.0 Died							5
3	p	p	17.5	457.5 Died								4
4		p	8.0	110.0	805.0	Dead						5.5
5		p	4.0	160.0	1235.0 Died							5
					Resistant strain							
1		p	1.5	24.0	220.0	*35.0**	65.0	215.0	870.0 1045.0 Died			9
2		p	0.5	17.0	222.5	535.0	*52.0**	210.0	965.0 Died			9
3	p	p	0.5	14.0	*3.0**	65.0	202.5	750.0	Dead			8.5
4				p	1.0	59.0	337.5	325.0	*28.0**	237.5	Dead	10.5
5		p	3.0	30.0	462.5	705.0	*10.0**	19.0	47.5	Dead		9.5

* Lowest count due to trypanolytic crisis.

reactions have set in. It is reasonable to assume that these did not appear suddenly on the day on which the relapse was evident but that they were already active on preceding days, possibly causing the destruction of parasites on a minor scale, which would give the same picture as a slower multiplication rate. A study of the figures shown in table 3 indicates that the predeath level of parasites of the drug-resistant strain might be slightly lower than that level in the normal strain, but this does not mean that the drug-resistant strain produces more toxic substances. Damage done to the host certainly must be cumulative, so that a strain which requires a longer time to kill its host may do with smaller numbers of parasites what another strain does in a shorter time with greater numbers of parasites. It is of interest that the 14 rats (table 1 and table 2) which succumbed to the arsenic-resistant strain following the progressive type of infection survived an average of 5.0 ± 0.63 days as compared with those infected with the normal strain, which survived 3.6 ± 0.06 days, while the 35 resistant rats suffering the relapse survived an average of 8.4 ± 0.12 days. However, 17 of these rats, for which the exact date of relapse was established (table 2), died 3.2 ± 0.13 days after the relapse occurred. Thus the relapse strain proved fatal to the rats in approximately the same time required by the normal strain to kill its host.

The survival time of animals infected with pathogenic trypanosomes is probably determined essentially by three factors: (1) the reproductive rate of the parasites, (2) the pathogenicity of the organism, and (3) the defense mechanism of the host. It is impossible to differentiate between these mechanisms in this case, but physiological studies reported elsewhere (von Brand et al, in press) have indicated a slightly lower metabolic level (lowered oxygen and sugar consumption, and pyruvate production) for the resistant strain than for the normal strain. This may indicate that an unknown toxic end product or metabolic function, which is damaging to the host, has been reduced. The infectivity, that is, the ability to become established in a host, as compared to pathogenicity, the amount of damage done to a host, was not significantly different in the two strains, as experiments with varying inoculums show (table 2). It should also be noted that the number of parasites required to produce a 100% infection was essentially the same in the two strains.

SUMMARY AND CONCLUSIONS

A comparison of the infectivity and pathogenicity of an arsenic-resistant strain of *Trypanosoma gambiense* with the normal strain from which it was developed shows that white mice, white rats, and guinea pigs infected with the resistant strain survived significantly longer than those infected with the normal strain.

Two patterns of infection were recognized, a progressive type and a relapsing type. Regardless of strain, all mice showed the progressive pattern and guinea pigs the relapsing pattern. However, rats infected with the normal strain showed only infection of the progressive type, while those infected with the resistant strain showed both types, with the relapsing pattern predominating. It is impossible to present a definite reason for the change in the pattern of infection which followed development of arsenic resistance in *T. gambiense*. Therefore, the two following explanations are presented: (1) the drug-resistant strain may have become less virulent by a reduced ability to multiply or to produce the same amount

of toxic products as the normal strain. This reduced virulence, in the case of the rats, would allow the immune mechanism of the body to build up to a greater degree than in the case of infection with the normal strain, thus leading to a trypanolytic crisis which in turn would prolong the infection. The same mechanism would lead to lengthening the time between two relapses and to more relapses, as seen in the guinea pigs. In mice the immune mechanism apparently could not be built up to the same extent; at least no crisis was evident, but infection with the resistant strain nevertheless progressed more slowly. (2) One can assume that during the development of arsenic resistance the metabolic mechanism has changed not only quantitatively but also qualitatively, leading to elaboration of different substances that change the immune response of the host. This also can explain the prolonged infections, the lower number of parasites, and the occurrence of relapses.

REFERENCES

von Brand, T. 1951, Metabolism of Trypanosomidae and Bodonidae. In Lwoff, A. Biochemistry and Physiology of Protozoa, vol. I: 177–234. New York, Academic Press, Inc.

von Brand, T., Tobie, E. J., Mehlman, B. and Weinbach, E. C. 1952, Observations on the metabolism of normal and arsenic-resistant *Trypanosoma gambiense*. J. Cell. & Comp. Physiol. In press.

Fulton, J. D. and Yorke, W. 1941, Studies in chemotherapy. XXVII. Further observations on the stability of drug-resistance in trypanosomes. Ann. Trop. Med. **35**: 221–227.

Jollos, V. 1921, Experimentelle Protistenstudien 1. Untersuchungen über Variabilität und Vererbung bei Infusorien. Arch. f. Protistenk. **43**: 1–222.

Reichenow, E. 1929, In Doflein-Reichenow, Lehrbuch der Protozoenkunde. ed. 5, Jena, Gustav Fischer.

Sonneborn, T. M. 1949, Beyond the gene. Am. Scientist, **37**: 33–59.

Yorke, W., Murgatroyd, F. and Hawking, F. 1931, Studies in chemotherapy. VI. The production of resistant strains by exposure of trypanosomes to reduced tryparsamide *in vitro*. Ann. Trop. Med. **25**: 521–544.

THE DIAGNOSIS OF STAPHYLOCOCCAL MASTITIS, WITH SPECIAL REFERENCE TO THE CHARACTERISTICS OF MASTITIS STAPHYLOCOCCI

L. W. SLANETZ AND CLARA H. BARTLEY

From the Department of Bacteriology, University of New Hampshire, Agricultural Experiment Station, Durham, New Hampshire

It is now recognized that staphylococci may cause either an acute or chronic type of bovine mastitis. In some herds they may produce a greater incidence of infection than *Streptococcus agalactiae*, and they are often of special concern in herds in which streptococcal mastitis has been eliminated. However, there is still lack of information as to the most suitable tests or criteria for the diagnosis of staphylococcal mastitis. More information is also needed regarding the characteristics of the staphylococci causing infection in the bovine udder.

During the past five years, we have made detailed studies on methods for the detection of mastitis staphylococci in milk samples obtained at monthly intervals from a herd maintained at approximately 100 milking cows. Samples from 305 different cows were tested. The herd was housed in two barns but milked by machines in the same milking parlor. During the last two years of study, the herd was free from *Str. agalactiae* mastitis.

In this study, we were particularly interested in establishing the characteristics of those staphylococci that cause an acute or a chronic type of mastitis infection. Thus, the type of hemolysis on sheep or cow blood agar, the coagulase reaction and the types of toxins produced by the staphylococci were correlated with the period and type of infection in the udder and with the leucocyte count of milk samples. The pathogenicity of selected strains of staphylococci and the toxicity of different types of toxins were determined by infusion into healthy quarters of experimental cows.

Received for publication September 11, 1952.

Scientific contribution no. 154 of the New Hampshire Agricultural Experiment Station.

This work was supported in part by grants from the American Cyanamid Company, Lederle Laboratories Division, Pearl River, New York.

The authors wish to thank Dr. Fred E. Allen, Mr. LeRoy R. Scarce and Mr. Francis M. Sirotnak for technical assistance.

METHODS

Collection of milk samples.—Before the collection of samples, the teats were carefully disinfected with a 1:1000 solution of mercuric chloride. The hands of the sample collector were also disinfected with this solution before collection of samples from each cow. About 20 ml of milk were then drawn from each quarter into a sterile, screw-cap bottle. During the latter part of the study, each bottle contained 1 ml of a 1:1000 dilution of sodium azide to inhibit the growth of possible contaminants. Foremilk samples were used; no milk was discarded before the samples were collected. The samples were kept on ice during transportation when unincubated samples were to be tested.

Examination of samples.—The following routine tests were made on all samples after incubation at 35 C for 18 hours.

1. *Microscopic test.*—Stained films were prepared by the Prescott and Breed technique for the determination of leucocyte count per ml of milk sample. A record was also made of the presence or absence of staphylococci or other bacteria in these preparations.

2. *Culture tests.*—One loopful (0.01 ml) of each milk sample was streaked over one-quarter of a blood agar plate, 1 plate being used for the 4 quarter samples from each cow. After incubation

for 36 to 48 hours at 35 C, the plates were examined for hemolytic staphylococci. Records were made as to the type of hemolysis produced on these plates. Following the terminology used by other investigators, a clear zone surrounding the colony or growth was designated as α, a zone of partial hemolysis or darkened red cells as β and the combination of a clear and a partial zone of hemolysis as α-β.

The basic medium contained 2% tryptose, 0.1% glucose, 0.5% sodium chloride and 2% agar at a pH of 7.0. While 5% citrated sheep blood was generally added to the medium, a number of tests were made on samples comparing sheep, cow and rabbit blood. The cow blood was obtained from young animals.

3. *Coagulase test.*—At each monthly sampling period, the hemolytic staphylococci developing on the blood agar plates were tested for their ability to coagulate rabbit citrated plasma or whole blood. During the first part of this study, pure cultures were isolated in yeast extract broth. After 18 hours incubation at 35 C, 0.1 ml of the culture was added to 0.5 ml of plasma. Coagulation was determined after 2 hours incubation at 37 C and 24 hours incubation at room temperature. Later it was found that a simpler and efficient technique was to suspend 1 large loopful of organisms taken directly from the blood plates in 0.1 ml of nutrient broth or saline in small tubes. To this suspension, 0.1 ml of citrated whole rabbit blood was added and the coagulation reaction determined after a 2-hour incubation period in a water bath at 37 C. A final reading was generally made after the tubes had incubated at room temperature for 24 hours.

Production and titration of toxins.—For the production of toxins, colonies chosen from the blood plates were inoculated into a starch broth containing 2% Bacto-tryptose, 1% Bacto-proteose peptone, 0.2% soluble starch, 0.5% sodium chloride, 0.5% glucose, 0.5% KH_2PO_4 and 0.5% K_2HPO_4. The pH was adjusted to 7.2. After 18 to 24 hours incubation, 1 ml of the broth culture was spread over an agar medium with the same composition as above plus 1% agar in 100 or 150 mm Petri dishes. The cultures were incubated at 35 C in an atmosphere containing 25% carbon dioxide.

For routine titrations, the toxins were harvested after 48 hours incubation by pressing the material from the Petri dishes through three layers of cheesecloth followed by centrifugation. If sterile toxins were desired, the clear filtrates were passed through Selas filters. Otherwise they were titrated directly after centrifugation.

Toxins were titrated by making duplicate serial dilutions in saline from 1:2 to 1:16,384 in agglutination tubes, each tube containing 0.4 ml of the diluted toxin. One-tenth ml of a 1% saline suspension of washed sheep red blood cells was added to one series, and a similar suspension of rabbit cells to the second series. Hemolytic titers were determined after 1 hour of incubation in the water bath at 37 C and after incubation overnight at 8 C. For the detection of δ toxins, the filtrates were also titrated using horse red blood cells.

Preparation of antitoxins.—For the production of antitoxins, Selas filtered toxins were titrated by the tube method and formalin added to make 0.3% final concentration. The formalized toxins were then incubated until they no longer lysed rabbit cells. Beta toxins were used without detoxification. One ml of the toxoid or toxin was injected intravenously in rabbits every other day until 6 injections were given. Rabbits were usually kept over a period of months and booster doses of 1 ml were given a few days before they were bled.

RESULTS

Approximately 24,000 quarter samples were tested to compare the efficiency of different tests for the diagnosis of staphylococcal mastitis. The characteristics of about 9,000 strains of staphylococci isolated from the samples were studied.

Microscopic test.—It was definitely established that the presence of staphylococci causing bovine mastitis cannot be accurately determined by microscopic examination of incubated milk samples. Many samples reported as negative by this test were found to contain hemolytic, coagulase-positive staphylococci when 1 loopful (0.01 ml) of the incubated milk sample was smeared over blood agar; also, samples which were recorded as positive on the basis of the microscopic test (presence of typical staphylococci) were found to contain no hemolytic staphylococci. Schalm (1948) reported similar results.

Culture tests.—The comparative value of sheep, cow and rabbit blood agar

plates for the detection of mastitis staphylococci was determined by correlating the type of hemolysis with the coagulase reaction, toxin production and pathogenicity of the staphylococci present in the milk samples.

In all cases, the blood agar plates were incubated at 35 C for 36 hours and examined for typical α, β or α-β zones of hemolysis. We found 36 hours incubation optimum, although good results can be obtained when the plates were incubated for 48 hours.

Either sheep or cow blood appears to be satisfactory for the identification of pathogenic staphylococci in milk samples. A 98% correlation between the two was found in 1656 quarter samples tested. The difference may well have been due to experimental error. Sheep blood was used routinely throughout this study. It is important that the blood contain no staphylococcal antitoxins, although we found this no problem if suitable animals are selected for bleeding. In some cases, more distinct zones of hemolysis were obtained when plates were prepared with washed sheep or cow red blood cells. Such blood plates also appeared to keep for a longer period of time in the refrigerator than the plates made from whole blood.

Agar plates prepared with rabbit blood were not satisfactory for the detection of pathogenic staphylococci in the milk samples. On the basis of tests on 1661 quarter samples using rabbit blood agar plates and sheep or cow blood agar plates, the correlation was only about 60%. Many staphylococci or micrococci which produced hemolysis on the rabbit blood showed no hemolysis on the sheep or cow blood agar plates. Such strains were found to be coagulase-negative. Another disadvantage is that the β type of hemolysis cannot be detected on the rabbit blood agar plates.

In order to compare the efficiency of unincubated and incubated milk samples for the diagnosis of staphylococcal mastitis, 1380 different samples were smeared on blood plates before and after incubation. Hemolytic coagulase-positive staphylococci were found in 411 of the unincubated quarter samples and in 565 of the incubated samples. Thus, in the unincubated samples, the organisms were missed in 154, or 27%, of the samples that were positive after incubation. Schalm (1948) obtained somewhat similar results. However, it was noted that only about a 7% discrepancy occurred in those samples with a leucocyte count of 1 million or more per ml. By use of blood agar plate counts, it was demonstrated that many more staphylococci are shed in the milk from quarters with a high leucocyte count. In unincubated samples with no leucocyte counts, the number of hemolytic, coagulase-positive staphylococci were often less than 100 per ml. In samples with leucocyte counts of 1 million or more, the bacterial counts usually averaged about 500 to 1000 per ml.

The above results would explain the discrepancy obtained with incubated and unincubated samples when only 0.01 ml of the milk sample is smeared on blood agar plates. Murphy (1943) suggested that the quarters could be considered "uninfected" if the milk samples contained less than 200 hemolytic staphylococci per ml. In our studies, cows with a well established chronic infection of a year or more duration sometimes shed less than 200 staphylococci per ml at different test periods. It should be noted, however, that for samples with leucocyte counts of less than 1 million, when the staphylococci were present in the incubated but not

in the unincubated samples, they sometimes were not found in such samples on subsequent tests. This perhaps indicates that in such cases the infection had not become established.

The argument has been offered that incubated samples may not be reliable for the detection of mastitis staphylococci since hemolytic staphylococci from the skin and hair of cows and even from the air of the dairy barns might get into the milk samples and multiply during incubation. To obtain some information on this question, the teats and lower portion of the udders of 75 cows were swabbed with a moistened, sterile cotton swab immediately before and 2 minutes after disinfection. Blood agar plates were also exposed in the barns for 5- to 15-minute periods. Hemolytic coagulase-positive staphylococci were isolated from the udders of 11 out of the 75 cows tested before disinfection but none from the swabs from the udders after disinfection. No hemolytic, coagulase-positive staphylococci could be isolated from 25 plates exposed in the dairy barns.

In general, no appreciable difficulty was experienced with contamination in incubated samples which were carefully collected. However, during the latter part of this study, the growth of air contaminants was practically eliminated by adding sodium azide to the sample bottles to make a final concentration

FIG. 1.—Types of hemolysis produced on sheep blood agar by staphylococci isolated from the udders of cows. 1, β hemolysis; 2, α hemolysis; 3, α–β hemolysis; and 4, weak hemolysis. Cultures 1, 2 and 3 are coagulase-positive; culture 4 is coagulase-negative.

of 1:20,000 in the milk sample. The concentration of sodium azide used produced no apparent inhibition of the growth of the staphylococci in the incubated milk samples.

Mediums described by Chapman (1946 and 1948) for the identification of pathogenic staphylococci had no advantage over blood agar and had the definite disadvantage of offering no information on the types of hemolysins or toxins produced by the organisms.

Coagulase tests.—Chapman et al (1934), Cruickshank et al (1937), Chapman et al (1938), Cowan (1938), Plastridge et al (1939), Fairbrother (1940), Slanetz et al (1945), Schalm (1948) and others have demonstrated that the coagulase reaction is a valuable and accurate test for the identification of pathogenic staphylococci.

Over the 5-year period, approximately 9000 strains of staphylococci isolated from blood agar plates prepared from quarter milk samples were tested for their coagulase reaction. The results obtained were carefully correlated with the type of hemolysis produced on the blood agar plates and, for many of the strains, with the type of toxins they formed. Records were also kept on the type and permanencey of the infection the organisms produced in the udders of the test cows.

On the basis of the data collected, the following conclusion appears justified. All staphylococci from the milk samples are coagulase-positive if they produce an α, an α and β, or a β zone of hemolysis on sheep or cow blood agar. Cultures which produced small α zones of hemolysis (1 mm or less from edge of growth) were found to be coagulase-negative in practically 100 % of the cases. As will be indicated later, they were also found to be weak toxin producers. Two hundred and thirty-two strains of non-hemolytic micrococci isolated from the blood agar plates were also found to be coagulase-negative. The above results indicate that with proper experience, the coagulase-positive or pathogenic staphylococci can be detected on the basis of the type of hemolysis produced on the blood agar plates. Typical hemolytic zones produced by these staphylococci on sheep blood agar are shown in figure 1.

Toxin production.—The amounts and types of toxins produced by 818 strains of staphylococci isolated directly from blood agar plates were determined during this study. Of these, 423 strains produced α and β hemolysis, 183 strains produced β hemolysis, 89 strains produced α hemolysis and 123 strains were weakly hemolytic (less than 1 mm zone of hemolysis). All of these strains were coagulase-positive except the last 123 strains listed.

The types of toxins were identified chiefly on the basis of hemolysis of rabbit and sheep red blood cells. Alpha toxin alone was considered present in a filtrate if both rabbit and sheep cells were lysed and no marked increase in the hemolytic titer of the sheep cells occurred in the cold after incubation for 24 hours at refrigerator temperature (8 C). Alpha and beta toxins were listed as present when both rabbit and sheep red blood cells were lysed and a definite increase in the hemolytic titer of sheep red blood cell lysis developed after incubation in the cold for 24 hours. Beta toxin alone was considered present if lysis of sheep cells occurred after 24 hours incubation in the cold with no lysis of rabbit cells. Some strains were tested for their ability to produce δ toxin by titration of the filtrates, using horse red bood cells in addition to rabbit and sheep red blood cells. It should be noted that in the final identification of the

toxins, the above tests were correlated with the type of hemolysis produced by the organisms on blood agar plates and by use of specific antitoxins.

All except 6 (99.5%) of the 695 coagulase-positive staphylococci tested for toxins produced hemolytic titers of 1:128 or higher on either rabbit or sheep red blood cells. Many of the strains produced potent α and β toxins, the titers ranging from 1:1024 to 1:16,384, the highest dilution tested. On the basis of the tests used, none of the strains produced only α toxin. Ten of the 695 strains produced β toxin alone. All the remaining strains formed both α and β toxins.

Bryce and Routree (1936), Minnett (1936), Slanetz et al (1945), Christre et al (1946), Smith (1947), Elek and Levy (1950) and Pellet et al (1950) have also reported that staphylococci of animal origin usually produce both α and β toxins. In this study, a number of strains were also tested for their ability to produce the δ type of toxin described by William and Harper (1947), Elek and Levy (1950), Marks and Vaughan (1950), Marks (1950 and 1951). The majority of the strains tested formed the δ toxin as well as α and β toxins. Thus it appears that typical, pathogenic mastitis staphylococci generally produce α and β or α, β and δ toxins. Only occasionally is a strain encountered which forms only β toxin. It should be further noted that the type of toxin produced by a given strain could not be definitely predicted on the basis of the type of hemolysis produced on the blood agar plates. For example, 183 strains produced only β hemolysis of the sheep blood agar plates but 173 of these strains were found to produce both α and β toxins by the tube titration tests of toxin filtrates. Also, 68 cultures were recorded as forming only α hemolysis on the sheep blood agar plates. However, when the toxins produced by these strains were titrated, both α and β toxins were present.

None of the toxin filtrates from 123 weakly hemolytic, coagulase-negative strains produced toxin titers above 1:64 for either rabbit or sheep red blood cells. No toxins could be detected in the filtrates from some of the strains. This is further evidence that these organisms are nonpathogenic and can be disregarded as causative agents of bovine mastitis. Cowan (1938) also observed that only the coagulase-positive staphylococci formed toxins.

Antitoxin production.—As an aid in determining the types and characteristics of staphylococcal toxins, specific antitoxins were produced in rabbits, using toxoid or toxins from strains producing β toxin, α toxin and α and β toxin. Since no culture was isolated from the milk samples which produced only α toxin, a human strain (Wood 46) was used for obtaining this type of toxin.

Neutralization studies on the toxins produced by a number of various strains of staphylococci confirmed the results reported in the previous section on the types of toxins produced by mastitis staphylococci.

Leucocyte counts in relation to staphylococcal mastitis.—Leucocyte counts were made on all quarter samples tested during the 5-year period and correlated with the presence of hemolytic staphylococci in the samples and the period of infection in the cows. Some investigators feel that a positive diagnosis for staphylococcal mastitis should not be made unless hemolytic staphylococci are present and a leucocyte count of 1 million or more per ml of the milk samples is obtained.

In our studies, only 47% of the

quarters with a continuous infection for periods of 3 to 12 months gave leucocyte counts of 1 million or more per ml at each test period. Observations were also made on the leucocyte counts of samples collected at monthly intervals over a 12- to 36-month period from 56 quarters of 25 cows which were continuously infected or were shedding hemolytic, coagulase-positive staphylococci for periods of 1 year or more. The leucocyte counts often varied from 0 to 10 million or more at each monthly test period. However, the leucocyte count for practically all quarters was less than 1 million for several of the monthly test periods. Typical examples of the results obtained for 1 cow are given in figure 2.

It was noted that, once a leucocyte count of 1 million or more and hemolytic, coagulase-positive staphylococci were present in the milk at a test period, the infection often persisted thereafter. Thus, in a group of 25 cows selected at random from the herd under study, only 8 cases were observed over the 5-year period in which the leucocyte count was 1 million or more and the staphylococci were eliminated from the quarter without treatment. However, in 42

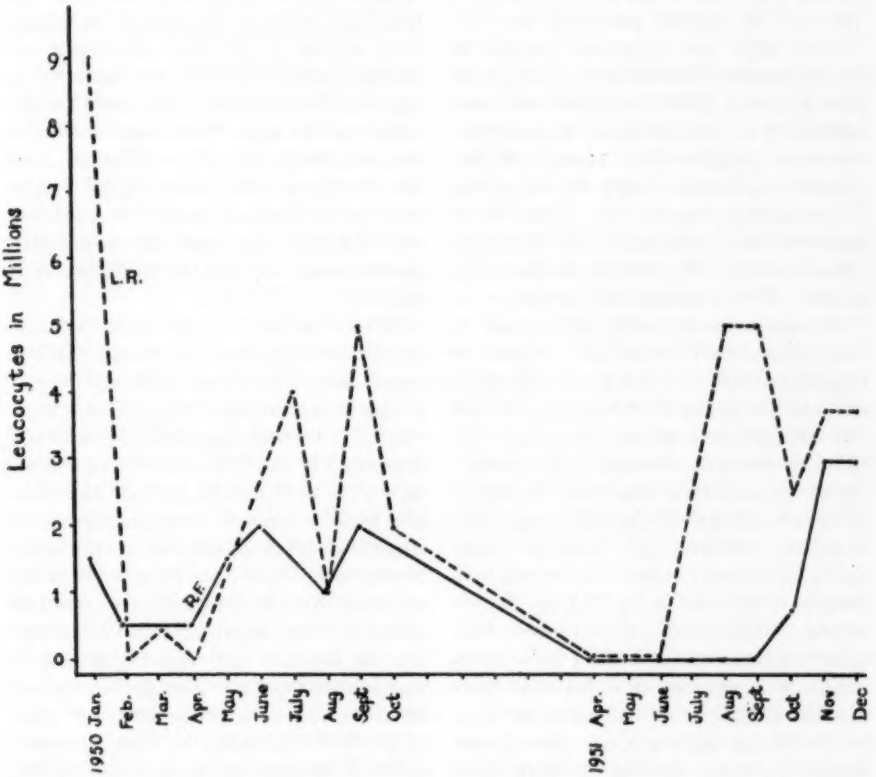


FIG. 2.—Fluctuations in leucocyte counts in milk samples collected at 1-month intervals from cow 384 over a 22-month period. Hemolytic, coagulase-positive staphylococci were present continually during this time in samples from the two quarters listed.

newly infected quarters with leucocytes counts which were below 1 million, the staphylococci disappeared at the next test period. In milk from 576 quarters which contained weakly hemolytic, coagulase-negative staphylococci, only 3.8% of the samples gave leucocyte counts of 1 million or more. In most cases these organisms disappeared from the quarters on subsequent tests.

Pigment production.—No systematic attempt was made to classify the many strains of staphylococci isolated during this study on the basis of pigment production. However, representative strains were tested at various times and the type of pigment produced was correlated with the coagulase reaction of the organisms. The cultures were grown on Chapman (1948) medium and were identified as *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) if the growth was orange, *Staphylococcus albus* (*Micrococcus pyogenes* var. *albus*) if no pigment was discerned and *Staphylococcus citreus* (*Micrococcus citreus*) if a lemon yellow pigment was formed.

In one series of tests, 161 strains of coagulase-positive and 126 strains of coagulase-negative staphylococci were checked for pigment formation. Of the 161 coagulase-positive strains, 143 (88.8%) were classed as *Staph. aureus*, 9 (5.6%) as *Staph. albus* and 9 (5.6%) as *Staph. citreus*. Of the 126 coagulase-negative cultures, 67 (53.1%) were classed as *Staph. aureus*, 56 (44.4%) as *Staph. albus*, and 3 (2.5%) as *Staph. citreus*. Similar results were obtained on other cultures studied. As others have noted, it is evident that pigment production cannot be employed as an indication of the pathogenicity of a given strain. It is of interest to note that *Staph. citreus* was isolated from cases of either a chronic or an acute staphylococcal mastitis in at least 15 different quarters in this herd.

Duration and spread of staphylococcal mastitis.—A number of quarters in this herd were chronically infected for as long as 3 to 5 years with the hemolytic, coagulase-positive staphylococci. Once the infection becomes established, it may exist as a subclinical condition, during which time only a few staphylococci and no leucocytes may be shed in the milk. However, any time during a lactation period, the infection may become acute, as evidenced by a high leucocyte count, abnormal milk or swelling of the quarter. The hemolytic, coagulase-positive staphylococci disappeared or were apparently eliminated from the quarters without treatment in about 10% of the cases. This generally occurred during the first few months of infection before leucocytes could be detected in the milk. In no instance did a coagulase-negative staphylococcus persist in the quarter samples for longer than a 3 to 6-month period; no evidence was obtained that such organisms produced cases of acute staphylococcal mastitis.

When the data on the incidence and spread of staphylococcal mastitis in the experimental herd were analyzed for the 5-year period, it was evident that a high rate of spread of this infection was occurring. Of the 248 test cows examined over a 38-month period, 161 were free from hemolytic, coagulase-positive staphylococci in all quarters at the start of the test. Only 3 of these cows were negative in all quarters after 12 months and only 1 was negative after 19 months. On the basis of individual quarters, it was found that the average spread of infection rate was about 20% per year in the 804 originally negative quarters studied. Studies are in progress to determine whether sterilization of the milking machine teat cups between each cow will reduce the spread of this infection in the herd.

TABLE 1.—*Results of injection of udders with selected strains of staphylococci.*

Quarter	Strain	Number of bacteria injected (24 hr. culture)	Clinical symptoms	Condition of milk	Duration of infection
			Cow 110		
RR	1*	300 million washed cells in 5 ml saline	None	Normal	None
RF	8		None	Normal	10 days
RR	1		None	Normal	3 months (then eliminated by treatment with aureomycin)
RF	8	5 billion bacteria (5 ml diluted broth culture	Quarter swollen for 72 hr.	Abnormal 72 hrs.	9 days
LF	71		Quarter swollen for 72 hr.	Abnormal 72 hrs.	9 days
LR	50		Quarter swollen for 4 days; fibrosis developed	Abnormal 4 days	9 days
RR	8	50 billion bacteria (5 ml undiluted broth culture)	Quarter swollen 48 hr.; temp. 105.4 F in 12 hr.	Abnormal 2 days	2 months (then eliminated by treatment with aureomycin)
			Cow 160		
RF	1		Normal	Normal	6 weeks (then eliminated by teratment with aureomycin)
RR	8	5 billion bacteria (5 ml diluted with culture)	Normal	Abnormal 3 days	6 weeks (then eliminated by treatment with aureomycin)
LF	50		Quarter swollen for 9 days	Abnormal 9 days	6 weeks (then eliminated by treatment with aureomycin)
LR	W46		Quarter highly swollen for 9 days	Abnormal 9 days	6 weeks (then eliminated by treatment with aureomycin)

* See text for type of toxins produced by each strain.

Virulence or pathogenicity studies in experimental cows.—The virulence or ability of selected strains of staphylococci to produce mastitis infection was tested by injection of the organisms through the teat canal into the quarters of young, lactating cows free from hemolytic, coagulase-positive staphylococci. The strains of staphylococci used for this experiment produced the following types of toxins: strain 1 β, strains 8 and 50 α-β-δ, strain 71 α-β and strain Wood 46 α-δ. All of these strains gave typical hemolysis on blood agar plates and were coagulase-positive. Strains 1, 8, 50 and 71 had been recently isolated from cases of mastitis infection. The Wood 46 strain was the classical α toxin-producing strain originally isolated from human sources. Table 1 shows the results of a series of injections in 2 cows with the above strains.

From these experiments, it is evident that large numbers of staphylococci are necessary to establish a permanent mastitis infection in certain cows. When 5 billion organisms were injected, active infections were established, although one cow (110) was able to eliminate the infection after 9 days even when such large numbers were injected. While a staphylococcus producing only β toxin (strain 1) established itself in the mammary tissue, no evidence of irritation or clinical symptoms of mastitis were observed other than an occasional leucocyte count of 1–2 million. It was demonstrated that marked irritation did develop when α toxin-producing strains were injected, even when these formed no β toxin. While a very severe reaction developed for a few days when 5 ml of a broth culture of strain 8 were injected, severe gangrenous mastitis did not develop in the infected quarter. Schalm (1944) reported that gangrenous mastitis was produced when 5 ml of pooled staphylococcus cultures were infused into quarters of lactating cows. This may be further evidence that there are differences in the susceptibility or resistance of individual cows to infection with staphylococci.

Toxin studies in experimental animals.—The toxicity or irritating effects of toxins produced by the same strains of staphylococci described in the previous section were tested by injection into quarters of experimental cows. The toxins were produced as outlined under

Methods and were made free from staphylococci by passage through Selas filters. The filtrates were then titrated and tested for sterility before injection via the teat canal into the quarters of the test cows.

On the first series of tests, 5 ml of an α-β-δ toxin from strain 8 (titer S, 1:512; R, 1:128; H, 1:32)* were injected into the RF quarter and 5 ml of a β toxin (S, 1:32; R, 0) in the LR quarter of cow 160. No hemolytic, coagulase-positive staphylococci had been detected in the milk of this cow on repeated tests over a 2-month period. Twelve hours after injection of the toxin, the RF quarter was markedly swollen and inflamed; the milk production was reduced by 50% from this quarter and the milk was discolored and contained clots. The temperature of the cow was 101.2 F. The milk contained a few clots for about 2 days following the injection but thereafter the quarter and milk returned to normal. No irritation could be demonstrated following the injection of the β toxin into the LR quarter and no increase in leucocyte count was observed in the milk. In further tests with the β toxin, 20 ml and 75 ml (titer S, 1:64; R, 0) were injected into different quarters of this cow. No irritation was produced by the 20 ml volume. The quarter receiving the 75 ml amount showed some swelling in 12 hours, the milk being of a watery consistency and containing a few fine clots. The temperature of the cow was normal but the milk flow was decreased by one-third. In two days the milk and quarter returned to normal.

To further study the effect of different staphylococcal toxins on the mammary tissue, the following toxins were injected into different quarters: (a) 5 ml toxin from strain Wood 46 (titer R, 1:256; S, 1:64; H, 1:8) diluted with 5 ml saline; (b) 20 ml β toxin from strain 1 (titer S, 1:256; R, 0; H, 0); (c) 5 ml of toxin (a) mixed with 5 ml of toxin (b); (d) 10 ml toxin from strain 8 (titer R, 1:1024; S, 1:512; H, 1:8); and (e) 15 ml toxin from strain 71 (R, 1:512; S, 1:128, R, 0).

To summarize briefly the results obtained, it was definitely demonstrated that the α toxin is highly irritating to the mammary tissue and when injected will produce typical clinical symptoms of acute mastitis. Some evidence was obtained that toxins from strains producing both α and δ toxins are more irritating than toxins from strains producing only α and β toxins. It was also definitely demonstrated that β toxin is nonirritating to the mammary tissue unless injected in very large amounts. However, it was found that β toxin may have a synergistic effect with δ toxin which increases the toxicity or irritating effects of such mixtures on the mammary tissue. This synergistic effect was also demonstrated by necrosis tests in rabbits and by an increase in the hemolytic zone on sheep blood agar plates. Thus strains which produce α-δ-β toxins may be more pathogenic or virulent than α-β-producing strains. The α toxins produced marked fibrosis of the mammary tissue.

The blood and milk serum of cow 160 was tested for staphylococcal antitoxins following the injections of the toxins. The milk serum contained no β- or δ-neutralizing antibodies but did contain appreciable amounts of α antitoxins. The blood serum completely neutralized the β and the α and δ toxins of strains 1, 8 and Wood 46. During the studies some of the results indicated that such antitoxins reduced the irritating effects of diluted toxins. This suggests that the

* Refers to hemolytic titer against sheep (S), rabbit (R), and horse (H) red blood cells.

antitoxins may play some role in the resistance of a cow to staphylococcal mastitis.

Cow 110, who had previously been infected artificially with various strains of staphylococci, was also injected with similar amounts and combinations of the toxins described above. All quarters were free of hemolytic, coagulase-positive staphylococci at the time the toxins were injected. However, the blood and milk serum of this cow did contain α and β antitoxins.

In general, the same results were obtained as have been described above for the tests with cow 160. The β toxin injected was nonirritating. The α toxins were highly irritating to the mammary tissue, producing typical symptoms of acute mastitis. When the β toxin was mixed with the α-δ toxin, increased toxicity was produced again, indicating that the β toxin may have an additive or synergistic effect presumably with the δ toxin. The antitoxins present in the blood and milk serum of this cow were apparently not sufficient to neutralize the large amounts of toxins injected.

Suggested procedures for the diagnosis of staphylococcal mastitis.—

Procedure A (incubated milk samples).

1. Incubate milk samples containing a 1:20,000 concentration of sodium azide for 18 hours at 35 C.
2. Prepare standard stained films for leucocyte counts.
3. Smear 1 loopful (0.01 ml) of each sample over one-fourth of a sheep or cow blood agar plate and note hemolysis after 36 to 48 hours incubation at 35 C.

Quarters should be considered positive for staphylococcal mastitis if colonies or growth of staphylococci with typical α, α-β, or β zones of hemolysis develop on the blood agar plates and if the milk shows a leucocyte count of 1 million or more per ml.

Quarters should be classed as suspect for staphylococcal mastitis if typical hemolytic staphylocci are found on the blood plates but the leucocyte count is less than 1 million. If the staphylococci are found in subsequent tests, then the quarters should be considered infected, regardless of the leucocyte count.

Procedure B (unincubated milk samples).

1. Smear 1 loopful (0.01 ml) of each unincubated milk sample over blood agar plates as outlined above.

Quarters should be considered infected or positive if typical hemolytic staphylococci develop on the blood agar plates as described for procedure A.

The comparative efficiency of the two procedures outlined above needs further study.

SUMMARY AND CONCLUSIONS

On the basis of the results obtained during this study, the following observations and conclusions appear justified:

1. Pathogenic staphylococci causing bovine mastitis can be detected and identified by smearing samples of milk over sheep or cow blood agar plates. Any colony or growth of staphylococci which produces a typical α, α-β, or β zone of hemolysis on these plates can be classified as a pathogenic mastitis staphylococcus. Repeated tests of large numbers of strains indicated that such organisms are always coagulase-positive and generally produce both α and β toxins of high potency. Those staphylococci which produce only narrow zones of α hemolysis (1 mm or less) are coagulase-negative, weak toxin producers and can be disregarded. The hemolytic, coagulase-positive staphylo-

cocci were found to produce either acute or chronic mastitis.

2. The leucocyte count cannot be relied upon to indicate the presence of a chronic staphylococcal mastitis infection. Milk samples from quarters with chronic infections of a year or more duration often show none or less than 1 million leucocytes per ml at different test periods. However, when a leucocyte count of 1 million or more per ml was obtained and hemolytic, coagulase-positive staphylococci were present, the quarter generally remained infected thereafter.

3. Pigment production was of little value for the identification of pathogenic mastitis staphylococci. It might be of interest to note that yellow pigment producing strains (*Staphylococcus citreus*) were found to produce acute or chronic mastitis in a few cases.

4. Staphylococci from cases of bovine mastitis usually produce α and β toxins. On the basis of the tests used, no strains were isolated which produced α toxin alone and only a very few strains which formed only β toxin. Many of the cultures tested also formed δ as well as α and β toxins.

5. The α type toxin produced by mastitis staphylococci is highly irritating to the mammary tissue and when injected into the udder via the teat canal will produce typical symptoms of acute mastitis. Beta toxin was essentially nontoxic. The β toxin mixed with δ toxin did appear to increase the toxicity of the δ toxin. Necrosis tests in rabbits supported the above observations.

6. Approximately 20% of the negative quarters in the experimental herd became infected with mastitis staphylococci each year during the 5-year period.

7. The strains of staphylococci which were hemolytic, coagulase-positive and formed α and β or α, β and δ toxins caused acute cases of mastitis when injected into quarters of experimental cows. Gangrenous mastitis did not develop even when 5 ml of an undiluted broth culture were injected. A strain which formed only β toxin did become established when injected into quarters of a test cow but produced no clinical evidence of infection.

8. Procedures for the diagnosis of staphylococcal mastitis are suggested.

REFERENCES

Bryce, L. M. and Roundtree, P. M. 1936, Beta toxin of staphylococci. J. Path. & Bact. **43**: 173–189.

Chapman, G. H. 1946, A single culture medium for selective isolation of plasma-coagulating staphylococci and for improved testing of chromogenesis, plasma coagulation, mannitol fermentation and the Stone reaction. J. Bact. **51**: 409–410.

———. 1948, An improved Stone medium for the isolation and testing of food poisoning staphylococci. Food Res. **13**(2): 100–105.

Chapman, G. H., Berens, C., Peters, A. and Curcio, L. 1934, Coagulase and hemolysin tests as measures of the pathogenicity of staphylococci. J. Bact. **28**: 343–363.

Chapman, G. H., Berens, C., Nelson, E. and Curcio, L. 1938, The differentiation of pathogenic staphylococci from non-pathogenic types. J. Bact. **35**: 311–333.

Christie, R., North, E. A. and Parkin, B. J. 1946, Criteria of pathogenicity in staphylococci. Australian J. Exper. Biol. & M. Sc. **24**: 73–78.

Cowan, S. T. 1938, The classification of staphylococci by precipitation and biological reactions. J. Path. & Bact. **46**: 31–45.

Cruickshank, R. 1937, Staphylocoagulase. J. Path. & Bact. **45**: 295–303.

Elek, S. D. and Levy, E. 1950, Distribution of hemolysins in pathogenic and non-pathogenic staphylococci. J. Path. & Bact. **62**: 541–544.

Fairbrother, R. W. 1940, Coagulase production as a criterion for the classification of the staphylococci. J. Path. & Bact. **50**: 83–88.

Marks, J. and Vaughan, A. C. T. 1950, Staphylococcal delta-hemolysin. J. Path. & Bact. **62**: 597–615.

Marks, J. 1951, Standardization of alpha-antitoxin with special references to anomalous

hemolysins including delta toxins. J. Hyg. **49**: 52–66.

———. 1952, Recognition of pathogenic staphylococci with notes on non-specific staphylococcal hemolysis. J. Path. & Bact. **64**: 175–186.

Minnett, F. C. 1936, Staphylococci from animals with particular reference to toxin production. J. Path. & Bact. **42**: 247–263.

Murphy, J. M. 1943, The general effect of staphylococcal infection on the biochemical composition of strict foremilk. Cornell Vet. **33**: 52–56.

Pillet, J., Isbir, S. and Mercier, P. 1950, Etude biologique des staphylocoques d'origine animale. Inst. Pasteur, **78**: 638–643.

Plastridge, W. N., Anderson, E. O., Williams, L. F. and Weirether, F. J. 1939, Characteristics of udder staphylococci. Storrs Agr. Exper. Sta. Bull. **231**: 1–60.

Schalm, O. W. 1944, Gangrenous mastitis in dairy cows. Vet. Med. **39**: 279–284.

———. 1948, Hotis test reactions by toxicogenic, coagulase-positive staphylococci. Am. J. Vet. Res. **IX, 30**: 11–19.

Slanetz, L. W., Howe, A. F. and MacLeod, H. P. 1945, Characteristics of staphylococci and staphylococcal toxins. Univ. N. H. Agric. Exper. Sta. Tech. Bull. #84.

Smith, H. W. 1947, The examination of staphylococci of animal origin with particular regard to the determination of criteria of pathogenicity. J. Comp. Path. & Therapeutics, **57**: 98.

Williams, R. E. and Harper, C. J. 1947, Staphylococcal hemolysins on sheep-blood agar with evidence for a fourth hemolysin. J. Path. & Bact. **59**: 69–97.

STUDIES ON IMMUNITY TO ASIATIC CHOLERA

VI. OBSERVATIONS ON THE RELATION OF ANTIBODY TO EFFECTIVE IMMUNITY TO EXPERIMENTAL ENTERIC CHOLERA, WITH A NOTE ON SULFONAMIDE THERAPY

WILLIAM BURROWS

From the Department of Bacteriology and Parasitology, The University of Chicago, Chicago 37, Illinois

Infection of the guinea pig by intragastric inoculation with *Vibrio cholerae* was described by Koch (1885) in support of the causal relation of this microorganism to Asiatic cholera in man. The infection was subsequently studied by Burrows, Elliott and Havens (1947) and shown to be a true enteric infection in that the vibrios were found to multiply in and remain largely confined to the lumen of the bowel. It was also observed that the experimental infection was modified by prior infection or by immunization with vaccine given parenterally, and that the modification was associated with the occurrence of agglutinating antibody in the feces. Though no doubt far from identical with cholera in man, this experimental guinea pig infection may be regarded as resembling the human disease in at least one important feature, i.e., the infection is for the most part localized in the bowel. It may be anticipated, then, that the immune mechanism operative in the guinea pig infection is similar to that of effective immunity in man. The possibility that the immunity is a consequence of the presence of coproantibody is an attractive one, and the analogy is at least not contradicted by the occurrence of coproantibody in man (Burrows and Havens, 1948). In any case, it seems probable that effective immunity is primarily a function of the effect of antibody on the microorganism without significant reinforcement by the phagocytic defense system.

The elucidation of the agglutinogenic structure of a group of cholera and noncholera vibrios (Burrows, Mather, McGann and Wagner, 1946) made it possible to examine the association of antibody to certain portions of the antigenic complex of the vibrios with protective activity. This was studied by Burrows, Mather, Elliott and Havens (1947), using the mouse protection test developed by Griffitts (1942 and 1944), in which both active and passive protection of the mouse is assayed by challenge inoculation of vibrios suspended in mucin. The results indicated that the antigen or antigens giving rise to protective antibody are associated with the heat stable fraction of the vibrios, but that protective antibody is not necessarily identical with O agglutinin. The mouse infection, a generalized bacteremia, is at best only remotely related to human cholera, and the present study was undertaken in the hope that the results might have some bearing on immunity to the naturally occurring disease in man.

The study reported here consisted of a series of experiments in which the effects of heterologous active immuniza-

Received for publication September 11, 1952.

This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

The writer is indebted to Dr. Marian E. Koshland, Miss Norma G. Deupree, Mr. Lawrence L. Ware, Miss Isabelle Havens, Miss Janet M. Lowe, and Miss Dorothy E. Moore, all of whom have contributed in some part to the study reported here.

tion, immunization with a purified toxin preparation previously shown to stimulate protective antibody formation in the rabbit (Burrows, Mather, Wagner and McGann, 1944), and homologous passive parenteral immunization on the enteric infection of the guinea pig were studied.

MATERIALS AND METHODS

Three vibrio strains were used. The strains of *V. cholerae* were NIH 41, an Ogawa strain, and NIH 35, an Inaba strain, both Indian strains that had been received from the National Institutes of Health in 1942, stored in lyophilized form, and maintained by weekly transfer on blood agar during these experiments. The third was a water vibrio of Chinese origin, Nanking 32/121, from the National Collection of Type Cultures. It was similarly kept in lyophilized culture and carried on blood agar for the present experiments. It was Greig-positive, cholera red-positive, Heiberg type I, and a member of O group II (cf. Burrows, Mather, Elliott and Wagner, 1946).

Immunizing antigens were prepared from 18- to 24-hour cultures on veal infusion agar, pH 8.0. The H-O antigens consisted of bacterial growth washed off in physiological salt solution containing 0.5% formalin, filtered through cotton to remove larger clumps, diluted in the same solution to contain 3 mg (dry weight)/ml, and allowed to stand at least overnight in the refrigerator before use. In the preparation of O antigen, the bacterial growth was suspended in physiological salt solution, filtered through cotton, diluted to contain 3 mg/ml, and refluxed for 2 hours as described elsewhere (Burrows, Mather, McGann and Wagner, 1946) to destroy completely the H antigen. Both kinds of antigens were diluted to barely perceptible turbidity for use as agglutinating antigens. The cholera antiserum was pooled hyperimmune rabbit serum prepared against the Ogawa O antigen, and had an agglutinin titer of 1:20,000 and a mouse protection titer of approximately 1×10^6; no preservative was added.

Enteric infection of the guinea pig was produced essentially as described elsewhere (Burrows, Elliott and Havens, 1947). The animals were guinea pigs approximately 300 g in weight, and were given 0.2 ml tincture of opium or 2 mg morphine sulfate in saline solution by intraperitoneal inoculation 10 to 20 minutes before intragastric inoculation with vibrio suspension in *M*/30 phosphate buffer, pH 8.0. Those animals which were actively immunized received 2 doses of 3 mg each of antigen by the intraperitoneal route 4 days apart. Passively immunized animals were given 2 ml of antiserum intraperitoneally. Urine and feces were taken as 24 hour specimens, separated by a bifurcated collecting funnel (Burrows and Havens, 1948), the latter collected in 30 ml of *M*/30 phosphate buffer of pH 8.0, on alternate days. After dispersion of the fecal material by shaking in a shaking machine, aliquots were removed for quantitative dilution in sterile saline blanks and cultured on Vedder and van Dam glycine-hemoglobin medium. Following removal of the samples for culture, formalin was added to 0.5%, the suspensions clarified at 10,000 r.p.m. in a Sorvall SS centrifuge, and the supernates used for titration of agglutinins. The urine specimens were discarded.

EXPERIMENTS

Active immunization with heterologous antigen.—Using both the active and passive mouse protection tests as criteria of immunogenic potency, it has been found that cross protection between the Inaba and Ogawa varieties of the cholera vibrio is essentially complete, that protective antibody is associated with antibody to the heat stable portion of the vibrio, and that protective antibody is separable from agglutinin in that cross protection is observed between vibrios having no common agglutinogens. Here the question of the relation of the antigen complex to effective immunity to the enteric infection was approached by active immunization with heterologous antigens followed by challenge by the intragastric route and bacteriological assay of the infection produced.

The Inaba strain NIH 35 and the Ogawa strain NIH 41 were used as both immunizing antigen and challenge inoculum, challenge being against the heterologous antigen in the experimental series and against the homologous antigen in the corresponding control series. Groups of six guinea pigs were immunized and challenged 4 days after the last inoculation of vaccine, the point of peak immune response as meas-

ured by fecal antibody titer. The challenge dose was 20 to 25 mg. Cross protection appeared to be complete in that the pattern of suppression of the infection by immunization with either heterologous antigen did not differ appreciably from that produced by homologous antigen in control series.

Similar experiments were carried out in which animals were immunized with O or H-O antigens prepared from Nanking 32/121, and challenged with NIH 41. The immunizing and challenge vibrios had a heat-labile antigen, 2, in common, but were completely unrelated in O antigen. Nanking 32/121 contained O antigen D present in some strains of *V. cholerae* but which was absent in the challenge strain. Nanking 32/121 was used in preference to other noncholera vibrios because it had a relatively high virulence for mice by the mucin technique—an LD_{50} of 245 as compared with an LD_{50} of 48 for NIH 41. Furthermore, by the passive mouse protection test, Nanking 32/121 hyperimmune serum gave a protective titer of about 17,000 against NIH 41 challenge as compared with 66,000 for the homologous antiserum to NIH 41; this was a part of the evidence supporting the conclusion that cross protection is demonstrable by the mouse test in the absence of agglutinogens common to the immunizing and challenge strains.

The results of two experiments in which groups of guinea pigs were immunized with O or H-O Nanking 32/121 antigen and subsequently challenged with doses of about 20 mg of NIH 41 are shown in figures 1 and 2, respectively, as percent cholera vibrios excreted together with fecal agglutinin titers to the immunizing and challenge antigens. In both of these series of animals the proportionate numbers of vibrios excreted were reduced over that expected for the nonimmune animal. Decline in numbers of vibrios was inversely related to the fecal agglutinin titer to the challenge strain, and was apparently unaffected by the presence of unrelated agglutinin to the immunizing antigen. There was clearly no difference between the O and H-O immunizing antigens as regards the behavior of the subsequent infection, though the latter included an H antigen common to the challenge strain. Thus, these results are in agreement with those given by the mouse protection test as regards the inability of antibody to the H antigen to modify the infection, and do not contradict the inference that cross protection attributable to a heat stable antigen other than O agglutinin may occur. It is, of course, true that this kind of experiment requires that the response to an essentially overwhelming infecting dose be modified and may be too crude to allow the definition of lesser degrees of partial immunity. The inverse kind of experiment, i.e., immunization with NIH 41 and challenge with Nanking 32/121, was possible because of the mouse virulence of the latter. This kind of experiment was carried out, immunizing with only the O antigen of NIH 41, and gave substantially the same results as those illustrated.

Active immunization with homologous endotoxin.—The preparation of purified cholera endotoxin by alcoholic extraction and precipitation with acetone has been described elsewhere (Burrows, 1944). Here the endotoxin of NIH 41 was prepared by extraction of the lyophilized O antigen with absolute alcohol for 2 hours in a Soxhlet apparatus. The activity was precipitated by the addition of 10 volumes of chilled acetone, and the precipitate sedimented by light centrifugation. It was dissolved in hot alcohol, the solution filtered to remove insoluble material, and again

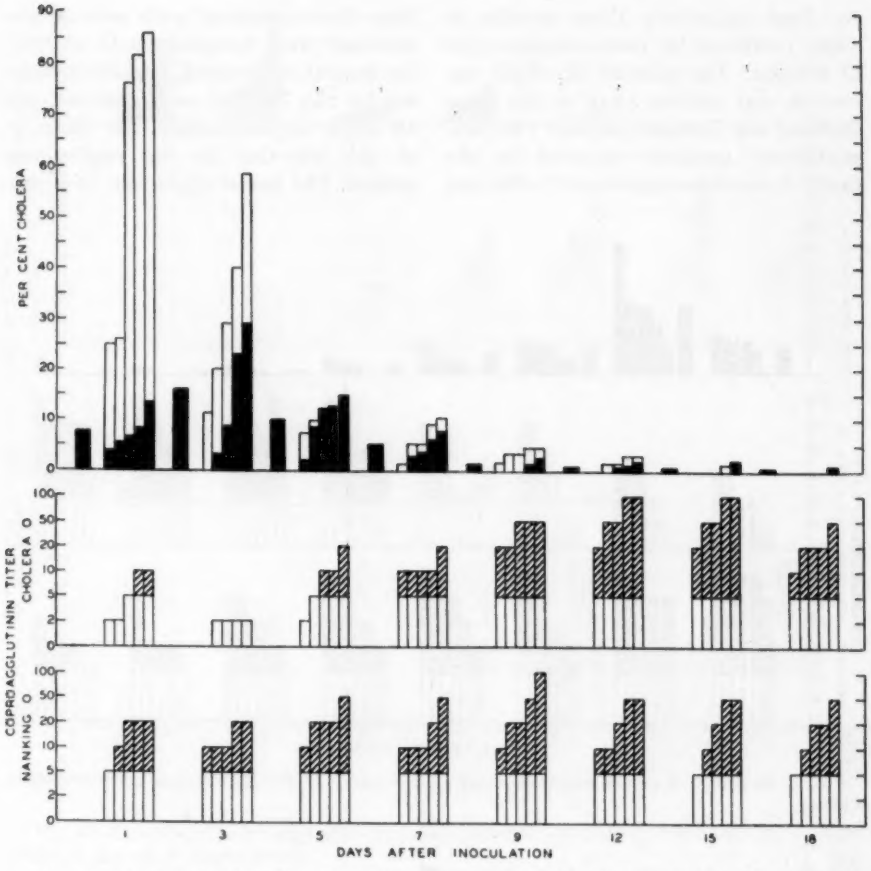


FIG. 1.—Infection of guinea pigs that had been immunized with Nanking 32/121 O antigen with *V. cholerae* NIH 41. In the upper portion of the graph the grouped solid bars represent individual animals, and the separate bars the average percent cholera vibrios excreted on the indicated days. Vibrio excretion by a control series of normal infected animals is shown for comparative purposes by the empty bars. In the lower portion of the graph the cross-hatched portions of the bars represent significant, i.e., above normal, agglutinin titers.

precipitated with acetone. After an additional precipitation, 19 volumes of physiological salt solution were added. Tne volume of alcohol had been adjusted so that the final solution contained endotoxin equivalent to 50 mg/ml of the vibrios from which it was prepared. This solution was opalescent and atoxic, but has been found to be immunogenic in the rabbit (Burrows, Mather, Wagner and McGann, 1944). In the present study it was used to immunize guinea pigs in 2 doses of 0.2 ml each, i.e., each dose contained the equivalent of 10 mg of vibrios, 4 days apart. A group of six animals so immunized was challenged 4 days after the second inoculation by the intragastric inoculation of 25 mg of NIH 41. The results of this experiment are shown in figure 3. The immunization gave an adequate immune response as indicated

by fecal agglutinin titers similar to those produced by immunization with O antigen. The pattern of vibrio excretion was neither that of the nonimmune nor immune animal. The proportionate numbers excreted in the first 3 days are not significantly different from those observed with animals immunized with homologous O antigen, but excretion persisted, actually increasing by the 7th day and continued for 18 days, approximately the duration of the infection in the nonimmune animal. The initial agglutinin titer pro-

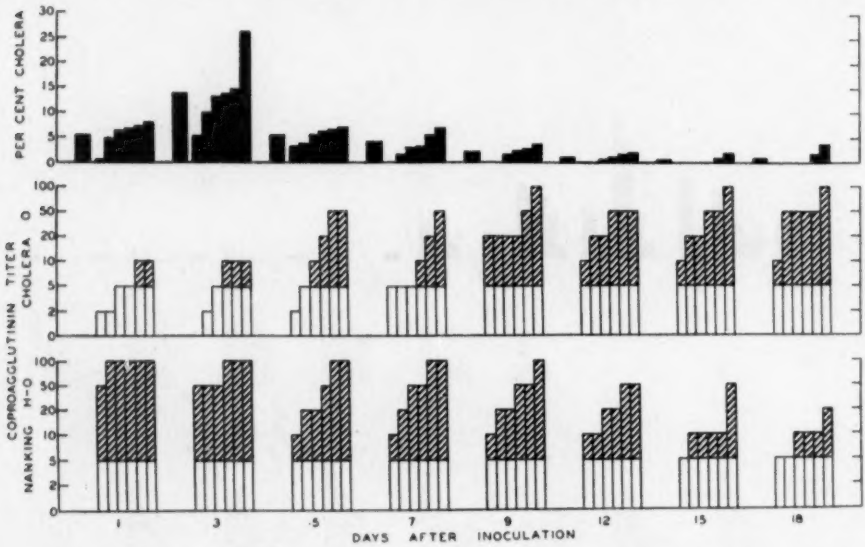


FIG. 2.—Infection of guinea pigs immunized with Nanking 32/121 H-O antigen with *V. cholerae* NIH 41.

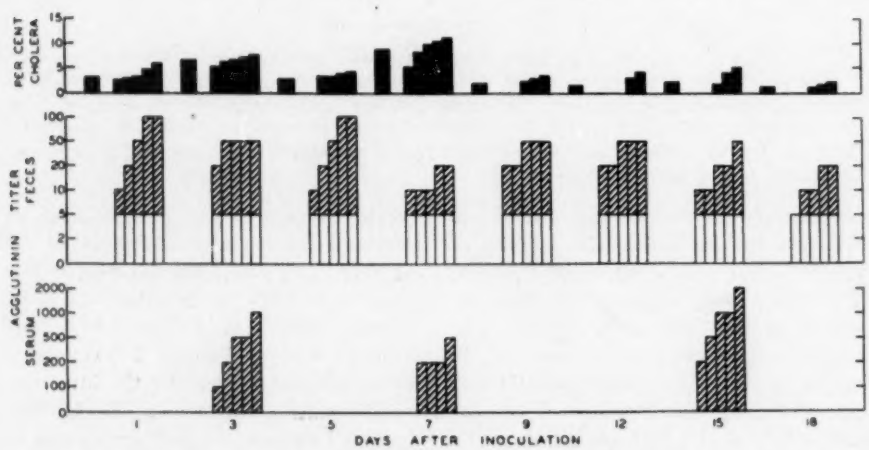


FIG. 3.—Infection of guinea pigs immunized with purified homologous endotoxin with *V. cholerae* NIH 41. Note the increase in vibrios excreted on the 7th day.

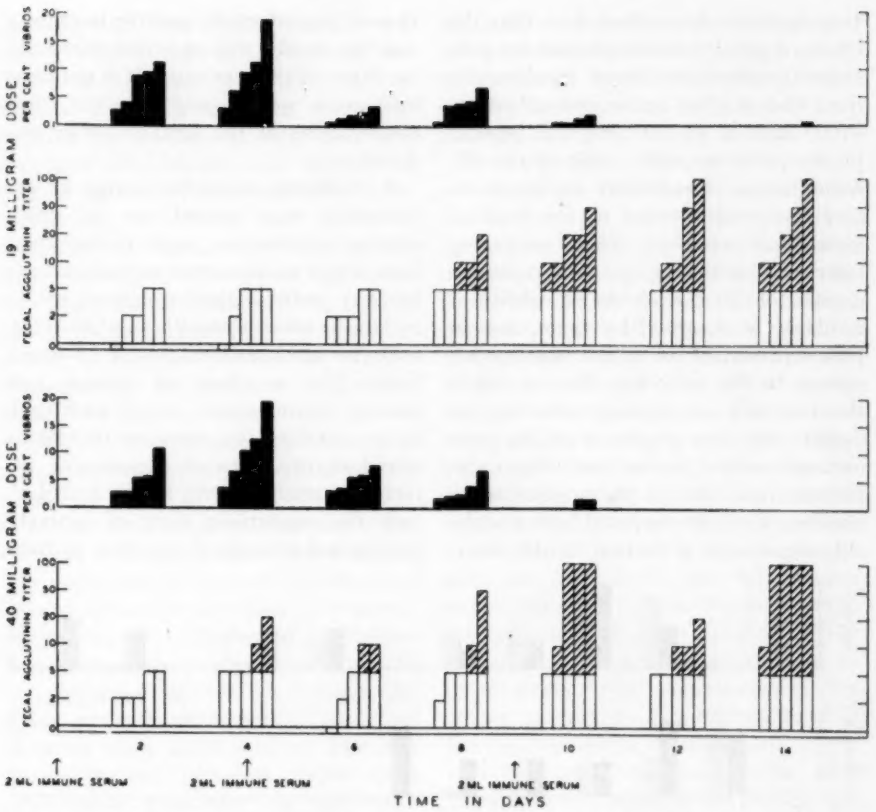


FIG. 4.—Infection of guinea pigs passively immunized with homologous antiserum with *V. cholerae* NIH 41 in doses of 19 and 40 mg.

duced by immunization with the endotoxin fell off during the early part of the infection and began to rise on the 9th day and coincided with a 90% reduction in percentage of vibrios excreted. It would appear, therefore, that while the preinfection immunization sharply reduced the numbers of vibrios excreted early in the infection, elimination of the infection was a consequence of an immune response to the challenge antigen.

Passive immunization.—Following parenteral inoculation of guinea pigs with antiserum of heterologous or homologous species origin, antibody has been found to be detectable in relatively high titer in fecal extracts in 24 hours (Burrows and Havens, 1948). The fecal titers equal or may exceed those obtainable by active immunization, and are roughly equivalent to titers expected on the assumption of dilution of the inoculated antibody in the total body weight of the recipient. A series of experiments was undertaken in which the effect of flooding the bowel with antibody by passive immunization begun prior to infection and carried on well into the period of infection was studied. The results of two representative experiments are illustrated in figure 4.

It is apparent from these data that the effect of passive immunization on subsequent infection differed significantly from that of prior active immunization. First, little or no antibody was present in the early stages in spite of the administration of sufficient antiserum to give appreciable titers in the feces of uninfected animals. That appearing later was probably in part a consequence of disappearance of vibrios if antibody is absorbed by them, and in part represented an active immune response to the infection. Second, while the intensity of the infection was reduced over that produced in the nonimmune animal, as indicated by a significant reduction in the proportionate number of vibrios excreted, and a probably significant reduction in the duration of the infection, passive immunity was not as effective as active immunity by either of these criteria. It is not clear how much weight may be given these observations in the light of other evidence.

A relatively extended series of experiments was carried out in which vibrios, antiserum, and complement were added to feces from normal animals midway in the collection period, singly and in the several possible combinations, and the specimens examined 12 hours later. The numbers of vibrios and amount of antiserum added were such as to simulate the numbers of vibrios found in the feces of nonimmune infected animals on the 1st to 2nd day, and the agglutinin titer of actively immunized animals at the peak of fecal

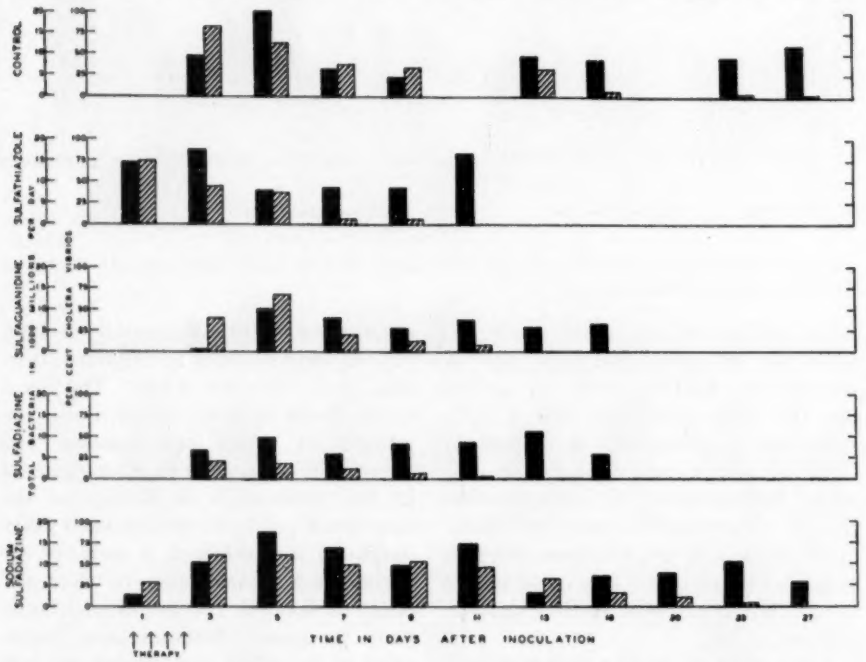


FIG. 5.—The effect of oral sulfonamide therapy on the infection of guinea pigs with *V. cholerae* NIH 41. The solid bars represent average numbers of total bacteria excreted per day, and the cross-hatched bars the observed percent of cholera vibrios. All data are averages for groups of six animals each.

antibody titer. The experiments showed that (1) there was no significant increase in numbers of vibrios in the absence of antiserum, but rather a decrease in that 60 to 90% were recoverable on culture; (2) agglutinin titer of antiserum was not reduced in the absence of vibrios; but (3) in the presence of vibrios plus antiserum, both numbers of vibrios and agglutinin titer were sharply reduced, the former by 70 to 80%; and (4) the apparent antibacterial effect of antiserum was not enhanced by the addition of complement. The failure to observe an increase in numbers of vibrios in the absence of antiserum may be attributed either to a combined effect of multiplication and death, or to a survival, without reproduction, of the majority of those added. The reduction in numbers in the presence of antiserum may thus have been a consequence of inhibition of multiplication if this were an important feature in the apparent survival, a bactericidal effect, or only an apparent reduction resulting from agglutination. The last seemed the more plausible in view of the pronounced reduction in agglutinin titer that suggested an absorption of agglutinin on the vibrios present. The feces were highly anticomplementary—10 units of complement per ml disappeared in 10 to 20 minutes—and this would account for the observed lack of effect of added complement. While such an admixture of vibrios, antiserum and complement with fecal pellets is hardly comparable to mixture within the fecal mass, it was of some interest that the results of experiments of this kind were similar to the effect of passive immunization on the infection. It seems possible, therefore, that the presence of antibody in the bowel of the passively immunized animal serves to give only an apparent reduction in numbers of vibrios excreted. Such a reduction, spurious or not, will, if persistent, reduce the duration of the infection since at best assay by culture of feces will show no more that 0.1% vibrios, and 1.0% is the more realistic figure.

Chemotherapy.—The results of a part of a study on sulfonamide prophylaxis and therapy of the enteric infection, carried out in another connection* and unpublished, are possibly related to an interpretation of the effect of passive immunization on the infection. A group of four experiments are shown in figure 5. Four sulfonamides, sulfathiazole, sodium sulfadiazine, sulfadiazine, and sulfaguanidine, were used as representing soluble and insoluble compounds. These were given by the intragastric route as 12.5% solutions or suspensions in two doses on each of two successive days, the day prior to infection and the day of infection. The first dose was 250 mg, and the remaining three doses 125 mg each. Feces were collected in 30 ml of physiological salt solution containing 10 mg percent *p*-aminobenzoic acid, it having been found that this concentration had no vibriocidal activity. The animals were infected with a 20 mg-dose of NIH 41. The antibacterial effect of the drugs is not necessarily confined to the cholera vibrio and may be differential, so both percent cholera vibrios and total bacteria were recorded. In figure 5 the solid bars represent total bacteria in 1000 millions per day, and the crosshatched bars the percent of these totals found to be cholera vibrios; the data are given as averages of observations of the six animals making up each group. Omitting detailed consideration as irrelevant to present purposes, the more effective of the com-

* This investigation was carried out for the most part by Dr. P. E. Harrison. The quoted experiment is a part of supplementary work done later, and is in substantial agreement with the remainder of the study.

pounds shown here, sulfadiazine and sulfaguanidine, appeared to reduce both the total numbers of bacteria and the percent of cholera vibrios, i.e., the cholera vibrio was differentially affected, and to shorten the duration of the infection. The effect of these drugs on the infection, then, was at least superficially closely similar to that of passive immunization, so much so that data on vibrio excretion are practically interchangeable.

DISCUSSION

An understanding of effective immunity to the enteric infection of the guinea pig with the cholera vibrio on an antibody basis is primarily dependent upon the manner in which antibody functions. While a certain parallelism between such an immunity and that to the parenteral infection of the mouse with vibrios suspended in mucin is to be expected, it need not follow that protective antibody titer determined by the mouse test is indicative of immunity to challenge by the intragastric route. It is self evident, too, that antibody titer measured by in vitro methods, such as agglutination, is likewise not necessarily a measure of effective immunity.

The extent to which the function of antibody in immunity to enteric infection can be approached by the use of the guinea pig infection is limited, both by the nature of the infection and by the character of the microorganisms. The significant features of the infection in this connection are (1) the focus of infection remains localized in the lumen of the bowel, (2) it is asymptomatic, and (3) its intensity is measured by the proportionate number of vibrios excreted in the feces. If the demonstrable immunity is accounted for on an antibody basis, it would follow that the antibody concerned is coproantibody though this has not been shown directly. Furthermore, the immunity measured is primarily antibacterial, directed toward elimination of the microorganism rather than toward the neutralization of toxicity leading to death. Study of the relation of qualitatively different fractions of the bacterial antigen to this kind of immunity is correspondingly dependent upon the occurrence of antigens in the bacteria in the case of active immunization, and upon the efficacy of passive immunization that, if effective, would allow the use of antiserums from which antibody fractions had been absorbed. The observations reported here lead, on the one hand, to several definite conclusions as to the relation of immunity to the enteric infection in the guinea pig to that against the generalized bacteremia occurring in the mouse following parenteral administration of vibrios in mucin, and, on the other, to conjectures inferred from observed discrepancies between the two.

The observation that effective immunity is associated with an immune response to the heat stable antigen of the cholera vibrio is not new. It was reported by Fairbrother (1928), who used parenteral infection of the guinea pig, and was later found to hold also for the mouse infection (Burrows, Mather, Elliott and Havens, 1947). The essentially complete cross protection between the Inaba and Ogawa varieties of the vibrio is also consistent with the results of the mouse protection test (cf. Griffitts, 1944; Ranta and Dolman, 1944; Burrows et al, 1947). Whether or not a cross immunity between strains unrelated in O agglutinogen occurs depends upon the significance given the partial reduction in vibrios excreted by the animals immunized with Nanking 32/121 and challenged with NIH 41. The cross protection observed in the mouse test was about 25% of that given

by homologous immunization. Assuming an average vibrio excretion by infected normal guinea pigs on the 2nd or 3rd day of about 50%, the average in both experiments illustrated in figures 1 and 2 is 15%, or approimately 30% of that expected in the absence of an immunity. This figure compares favorably with the results of the mouse protection test, but evaluation of the comparison is difficult. It may be concluded tentatively that the results of the guinea pig experiments described here are not inconsistent with those of the mouse test in support of the inference that antibody to a heat stable antigen unrelated to the O agglutinogen complex may be in part responsible for effective immunity.

A clear divergence between the enteric infection and the mouse test does occur with regard to the efficiency of purified endotoxin as an immunizing agent, and to the effects of passive immunization. The endotoxin preparation has been shown (Burrows, 1944) to be a highly efficient immunizing agent, both as to stimulation of protective antibody formation in the rabbit, and by the active mouse protection test. While the interpretation of data such as those shown in figure 3 is difficult, it is quite clear that the endotoxin preparation was, at best, an inferior antigen. This is possibly attributable to the differences between the two experimental infections. The asymptomatic, nonlethal character of the enteric infection in the guinea pig suggests that vibrio toxicity is not an important feature of it. On the other hand, the mouse infection may be regarded as one in which the vibrios are protected by mucin against a highly efficient normal defense mechanism, and the microorganisms multiply until a lethal amount of endotoxin has accumulated. The mouse protection test, then, may be considered to be one in which immunity is titrated against the virulence-enchancing property of mucin rather than the virulence of the microorganism. Under such circumstances toxicity may be of relatively great importance, and even a few-fold immunity to endotoxin might well magnify an inferior antibacterial immunity arising from contamination of the endotoxin preparation with small amounts of other antigen. That there was an antibacterial immunity may be inferred from the small reduction in numbers of vibrios excreted by the infected guinea pigs.

The behavior of the enteric infection in the passively immunized animal may be related to the nature of an effective immune response, but in just what way is not completely clear. Passive immunity is highly effective by the mouse protection test, and the LD_{50} ratio of immune to normal control animals is of the order of 1×10^4 to 1×10^6. In contrast, passive immunization served only to reduce somewhat the number of vibrios excreted by the infected guinea pig. This antibacterial effect may, as suggested above, be spurious, in that the reduction in numbers was a consequence of agglutination. However, the closely similar effect of therapy with sulfonamides, substances known to be primarily bacteriostatic, may be taken to suggest that multiplication of the vibrios is inhibited in the passively immunized animal. In the absence of direct evidence of bacteriostatic activity of antibody, it is more reasonable to assume that the apparent inhibitory effect of flooding the bowel with antibody by passive immunization resulted from a limited bactericidal activity in the presence of inadequate amounts of complement.

To carry the last inference further, the efficacy of passive immunity in the mouse and of active immunity to the enteric infection in the guinea pig,

and the inadequacy of passive immunity to the enteric infection, may be reconciled by assuming that the vibriocidal and vibriolytic properties of antibody make possible effective immunity, and that complement is required for this activity. In the passively immunized mouse antibody is temporarily stored and available as complement is continuously supplied. In the actively immunized guinea pig the secretion of immune globulin into the bowel is hardly a selective process; rather, it is probable that the several components of plasma, including complement, are secreted together, and antibody and complement are simultaneously available for reaction with the vibrios. While the activity of an antibody-complement lytic mechanism functional in the bowel has not been directly demonstrated, it is of more than passing interest that it has been suggested independently by Raffel (1949).

It remains to account for the absence of agglutinin in the feces of passively immunized infected guinea pigs in contrast with the presence of agglutinin when the animals have been actively immunized. It has been observed by the writer in unpublished experiments that, following immune lysis in vitro, the cell substance of the cholera vibrio autolyzes rapidly as evidenced by a shift of phosphorus from the lipid, protein and nucleoprotein fractions to the acid-soluble fraction. It may be suggested here that, following lysis by an antibody-complement system in the bowel, autolytic decomposition of the antigen liberates antibody which then becomes titratable as agglutinin.

CONCLUSIONS

The following conclusions regarding immunity to the enteric infection in the guinea pig may be drawn from the observations reported here:

1. Cross immunity between the Inaba and Ogawa types of the cholera vibrio was substantially complete.

2. A partial cross protection, similar to that demonstrable by the mouse test, occurred between vibrio strains having no O agglutinogens in common.

3. Active immunization with purified endotoxin resulted in an immunity inferior to that produced by active immunization with O antigen.

4. Homologous passive immunity was considerably less effective than active immunity.

5. Oral therapy with sulfadiazine or sulfaguanidine was almost as effective as active immunization; sulfathiazole had less effect, and sodium sulfadiazine practically none.

REFERENCES

Burrows, W. 1944, The endotoxin of the cholera vibrio: isolation and properties. Proc. Soc. Exper. Biol. & Med. **57**: 306–308.

Burrows, W., Elliott, M. E. and Havens, I. 1947, Studies on immunity to Asiatic cholera. IV. The excretion of coproantibody in experimental enteric cholera in the guinea pig. J. Infect. Dis. **81**: 261–281.

Burrows, W. and Havens, I. 1949, Studies on immunity to Asiatic cholera. V. The absorption of immune globulin from the bowel and its excretion in the urine and feces of experimental animals and human volunteers. J. Infect. Dis. **82**: 231–250.

Burrows, W., Mather, A. N., Elliott, M. E. and Wagner, S. M. 1946, Studies on immunity to Asiatic cholera. I. Introduction. J. Infect. Dis. **79**: 150–167.

Burrows, W., Mather, A. N., Elliott, M. E. and Havens, I. 1947, Studies on immunity to Asiatic cholera. III. The mouse protection test. J. Infect. Dis. **81**: 157–174.

Burrows, W., Mather, A. N., McGann, V. G. and Wagner, S. M. 1946, Studies on immunity to Asiatic cholera. II. The O and H antigenic structure of the cholera and related vibrios. J. Infect. Dis. **79**: 168–197.

Burrows, W., Mather, A. N., Wagner, S. M. and McGann, V. G. 1944. The endotoxin of the cholera vibrio: immunological properties. Proc. Soc. Exper. Biol. & Med. **57**: 308–311.

Fairbrother, R. W. 1928, The structure of *V.*

cholerae with reference to its immunizing properties. Brit. J. Exper. Path. **9**: 89–96.

Griffitts, J. J. 1942, The use of mucin in experimental infections of mice with *Vibrio cholerae*. Pub. Health Rep. **57**: 707–710.

———. 1944, Mouse protective antibodies in human serums following injections with cholera vaccine. Pub. Health Rep. **59**: 1374–1384.

Koch, R. 1885. Zweite Konferenz zur Erörterung der Cholerafrage. Deutsche med. Wchnschr. no. 37A (September 12), pp. 1–60.

Raffel, S. 1949, Types of acquired immunity against infectious disease. Ann. Rev. Microbiol. **3**: 221–264.

Ranta, L. E. and Dolman, C. E. 1944, Observations on cholera vaccine. Canadian J. Pub. Health, **34**: 26–37.

STUDIES ON IMMUNITY TO ASIATIC CHOLERA

VII. PROPHYLACTIC IMMUNITY TO EXPERIMENTAL ENTERIC CHOLERA

WILLIAM BURROWS AND LAWRENCE L. WARE

From the Department of Bacteriology and Parasitology, The University of Chicago, Chicago 37, Illinois

It has been reported in earlier papers of this series that antibody is excreted in the feces of guinea pigs during the course of experimental enteric infection with the cholera vibrio (Burrows, Elliott and Havens, 1947), and also in the feces following immunization with parenterally administered vaccine (Burrows, 1948; Burrows and Havens, 1948). The immune response appeared to bear a relation to the infection in that the number of vibrios excreted by the infected normal animal was reduced coincident with the appearance of coproantibody, and prior immunization, active or passive (Burrows, 1953), resulted in a reduction in the numbers excreted, especially in the early days of infection. Immunization did not, however, suffice to prevent infection by the relatively large doses, 20 mg dry weight, given by intragastric inoculation, and doses of this order may perhaps be regarded as overwhelming.

The question of immunity to enteric infection has been largely one of an effective prophylactic immunity. Asiatic cholera is the only enteric disease of man in which the efficiency of active immunization against the naturally occurring disease has been studied in extensive field tests. Here it seems clear that prior immunization does not appreciably modify the severity of subsequently acquired disease in that case fatality rates are not reduced, but a significant degree of prophylactic immunity is produced (Adiseshan, Pandit and Venkatraman, 1947). In man the observed prophylatic immunity is a clinical immunity, i.e., an immunity to infection accompanied by clinical symptoms, and may or may not be identical with immunity to inapparent infection.

Other than cholera, few if any of the enteric diseases of man may be reproduced in experimental animals in truly enteric form, that is to say, a form in which the infection is confined essentially to the bowel without generalized invasion of the tissues. The significant feature of this kind of infection is the proliferation of the microorganisms outside the tissues of the body, viz., in the lumen of the bowel, and hence without exposure to serum and tissue antibody, at least in the early stages when the infection is established. A prophylactic immunity having an antibody basis would seem, therefore, to be related to the presence of coproantibody. Experimental enteric cholera in the guinea pig serves as a model infection, and provides an opportunity to examine the prophylactic effect of immunization. Since the experimental infection is not accompanied by overt symptoms, the criterion of infection is the excretion of vibrios in sufficiently large numbers as to indicate multiplication of those inoculated, and a prophylactic immunity assayed by means of this infection is necessarily a measure of prophylaxis of infection rather than disease. The

Received for publication September 11, 1952.

This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

experiments reported here, and elsewhere in preliminary form (Burrows and Ware, 1949), were designed to show whether or not such a prophylactic immunity can be produced and, if so, its magnitude and duration.

MATERIALS AND METHODS

An Ogawa strain of *Vibrio cholerae*, NIH 41, was used exclusively in this study. It was received from the National Institute of Health in 1942, kept in stock as a lyophilized culture, and maintained by weekly transfer on blood agar during the course of the work reported here. Both O vaccine and challenge inoculums were prepared from 18- to 24-hour cultures on veal infusion agar, pH 8.0. The bacterial growth was washed off in physiological salt solution and filtered through cotton. For the preparation of O vaccine the suspension was refluxed for 2 hours to destroy the H antigen, and diluted to contain 1 mg/ml. This antigen was used both for active immunization of guinea pigs subsequently challenged, and for the immunization of rabbits in the preparation of antiserums. The fresh saline suspension was appropriately diluted in *M*/30 phosphate buffer just prior to use as a challenge inoculum. The doses given could be accurately measured over the range 0.01 to 1.0 mg/ml in the Klett-Summerson photoelectric colorimeter, using a no. 42 filter.

The guinea pigs used were uniformly 300 to 350 g in weight. The methods of challenge inoculation by the intragastric route following the administration of tincture of opium or morphine sulfate, of collection of excreta with separation of urine and feces, of the titration of agglutinating antibody in fecal extracts, and of the assay of the infection by culture of the fecal emulsion have been described in previous reports (Burrows and Havens, 1948; Burrows, 1953). Those animals which were actively immunized received 3 doses of O vaccine of 1 mg each by intraperitoneal inoculation 4 days apart. Passively immunized animals were given 2 ml of pooled hyperimmune rabbit serum, agglutinin titer of 1:10,000 to 1:20,000, intraperitoneally or by intragastric inoculation 24 hours before inoculation with vibrio suspension. In the case of intragastric immunization, tincture of opium or morphine was given parenterally prior to inoculation.

In the beginning it was supposed that the absolute numbers of cholera vibrios excreted by infected animals would be of importance in that only those excreting more than had been given by inoculation could be regarded as infected. It was almost immediately apparent, however, that if vibrios were excreted at all, they were excreted in numbers many thousand-fold that of the largest inoculum used; in one of the early experiments in which the absolute numbers were determined following infection with a 1 mg dose, the fold increase in vibrios recovered over those inoculated was about 80,000. The pattern of vibrio excretion following infection with minimal doses differed appreciably from that observed with doses of 7 to 20 mg and more in that the vibrios did not make up such a large percentage of the fecal flora—5 to 10% in contrast to 10 to 70 percent—and in that the infection was of somewhat shorter duration—3 to 10 days in contrast to persistence of infection in a few animals for as long as 3 weeks following larger doses. It was also observed that, with very small doses, excretion was often irregular, and was sometimes delayed as much as 3 days. An animal was counted as infected if at any time during the period of observation, not less than 1 week and 4 successive specimens, detectable numbers of vibrios were excreted. This criterion of infection is arbitrary in two respects, viz., numbers smaller than those detectable, 0.1% of the total fecal flora cultivable on the medium used, could be a multiple of the inoculum and yet be missed, and excretion may have occurred during periods when specimens were not taken. While the results are thus subject to absolute error, they have relative validity and may be legitimately compared with one another.

A dose producing infection in 50% of the animals inoculated may be calculated by interpolation in the usual ways. The experimental procedure here consisted of roughly approximating the end point by inoculation of pairs of animals with graded doses, and then infecting groups of 6 animals each with not less than 3 graded doses falling within the range of 0 to 100% infection. Variability is reduced to reasonable proportions in groups of this size (cf. Burrows, Elliott and Havens, 1947), and there was little to be gained by using larger numbers of animals. The median infecting dose, here designated the ID_{50} dose, was calculated by inverse probability:

$$p = \frac{n+1}{n+m+2}$$

where p is the probability of infection with a given dose, n is the accumulated infections, and m is the accumulated numbers of those not infected. The probabilities were plotted on arithmetic probability paper, a straight line fitted by inspection, and the ID_{50} determined graphically.

EXPERIMENTS

Preliminary experiments indicated that the ID_{50} of this strain of *V. cholerae* for the guinea pig was about 0.1 mg on a dry weight basis. It was titrated more precisely in a series of groups of animals receiving doses of 0.01 mg, 0.05 mg, 0.07 mg, 0.10 mg, 0.75 mg, and 1.00 mg. The results are illustrated in figure 1, and the character of the infection in normal animals is apparent. In this series all of the animals were infected by a dose of 1.0 mg and none were infected by doses of 0.05 mg and less; the latter are omitted from figure 1. On the basis of these data the ID_{50} was calculated to be 0.08 mg, with a standard deviation of 0.02. The coproantibody response to the dose of 1.0 mg was appreciable, and by the 5th day titers ranged from 1:10 to 1:50 in the animals in this group. It was progressively less with decrease in dosage, being barely significant with 0.05 mg though some slight response was still apparent with a dose of 0.01 mg, 2 of the 6 animals showing titers of 1:20 while the remainder were in the normal range of 1:5 or less. There was, of course, no significant titer at the time of inoculation.

Active immunity.—Animals were immunized with O vaccine and infected 4 days after the last inoculation, at the time of maximal coproantibody titer. The results of a titration over a dose range of 0.5 to 3.0 mg are shown in figure 2, except that failure to infect with a dose of 0.5 mg is not included. The ID_{50} was calculated to be 1.13 mg, an increase of 14.1-fold over the ID_{50} dose for normal animals. Here, and in subsequent experiments, coproagglutinin titers of a group receiving near the calculated ID_{50} dose are included, and were not significantly different from those of groups of animals receiving other doses. It is apparent that the coproantibody response was at its height at the time of infection and its decline was not as marked in these infected animals as has been observed in immunized but noninfected animals (Burrows, Elliott and Havens, 1947). The effect of immunization on the infection is shown as an increase in the ID_{50} dose, but there appears to be no

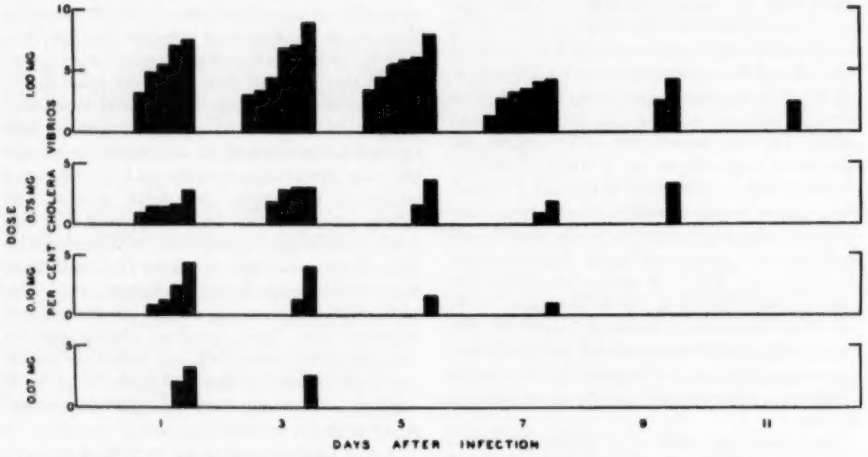


FIG. 1.—Infection of groups of 6 normal guinea pigs with graded minimal doses of *V. cholerae*. The ID_{50} dose is 0.08 mg.

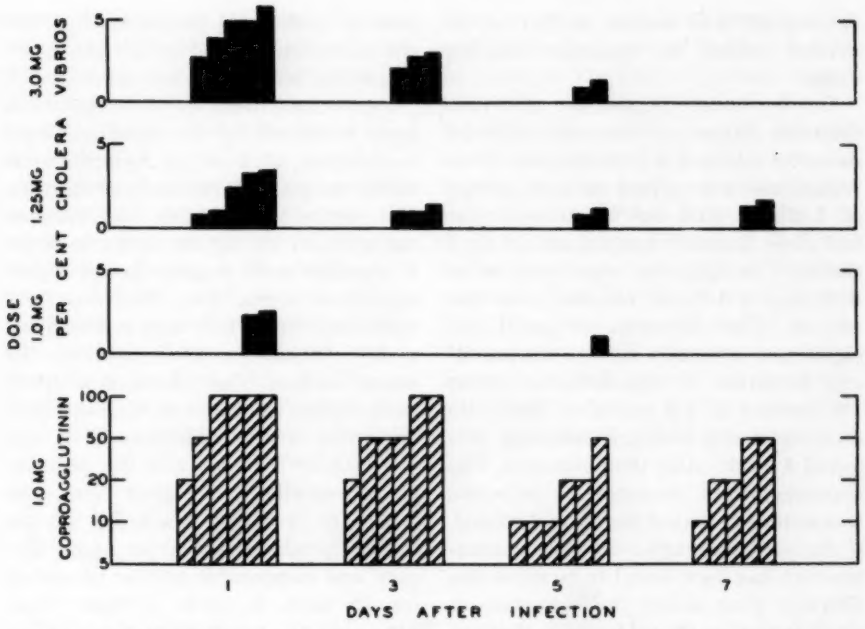


FIG. 2.—Infection of groups of 6 actively immunized guinea pigs 4 days after the last of 3 inoculations of cholera O vaccine. Coproagglutinin titer is at a peak and the light infection with a dose of 1 mg contrasts with that produced by the same dose in the normal animal (fig. 1). The ID_{50} dose is 1.13 mg.

essential difference in the bacteriological character of the infection around this end point, as indicated by comparison with doses of 0.1 and 0.25 mg in figure 1.

It has been reported elsewhere (Burrows, Elliott and Havens, 1947; Burrows and Havens, 1948) that the coproantibody response of the guinea pig is rapid, much more so than in man. The titers decline equally rapidly with only small titers apparent at 14 days and only traces, if any, of significant titer, by 28 days. Nevertheless, the residual titer at 14 days and/or an accelerated response to the booster effect of infection at this time resulted in the immune pattern of vibrio excretion following infection with large doses such as 20 mg. The possibility that the observed decline in coproantibody titer might, however, be associated with a decline in prophylactic immunity was investigated by delaying infection until 14 days after the last inoculation of vaccine. The results of a titration over the dose range 0.75 to 1.25 mg are illustrated in figure 3; failure to infect with a dose of 0.5 mg is not shown. At the time of inoculation only small residual coproagglutinin titers were found, and even on the 1st day of infection only 2 animals of the 6 showed significant, though small, titers. Unlike the infection produced by overwhelming doses, that resulting from minimal doses shows a decline in immunity in a reduction of the ID_{50} dose to 0.70 mg, an 8.7-fold increase over that for the normal animal, indicating a decline in prophylactic immunity of 48% of that demonstrable 4 days after immunization. The coproagglutinin response to the infection was

not marked and similar to that of the normal animal to minimal infecting doses.

In view of the persistence of a considerable degree of immunity over 14 days, an additional titration, not illustrated here, was carried out over a range of 0.075 to 3.00 mg in animals that had been actively immunized 28 days earlier. The ID_{50} dose was found to be 0.15 mg, a 1.9-fold increase over the normal. The difference is small but significant in that its ratio to the standard deviation of the difference from the normal is 3.5 and $P=0.0005$, indicating that a residual immunity persisted 4 weeks after immunization. The coproagglutinin response to infection was similar to that of the normal animal.

Passive immunity.—Passive immunization has been found to be much less effective than active immunization in the infection produced by large doses of the cholera vibrio (Burrows, 1953). Here the possible prophylactic effect of passive parenteral immunization and the administration of antiserum by intragastric inoculation was investigated.

Significant titers of coproagglutinin were produced by the intraperitoneal inoculation of 2 ml of hyperimmune rabbit serum 24 hours before infection. The results of a titration over the dose range 0.1 to 1.0 mg are shown in figure 4, together with representative coproagglutinin titers. The ID_{50} dose was calculated to be 0.20 mg, representing a 2.5-fold increase over that for the normal animal. The difference is significant since its ratio to the standard deviation of the difference is 6 and $P=2.0\times10^{-7}$. In spite of the presence of coproantibody to good titer, the immunity is clearly markedly inferior to that produced by active immunization, and comparable to that persisting for 28 days in such animals, when little or no coproagglutinin titer is demonstrable. When infection was delayed 14 days after the parenteral inoc-

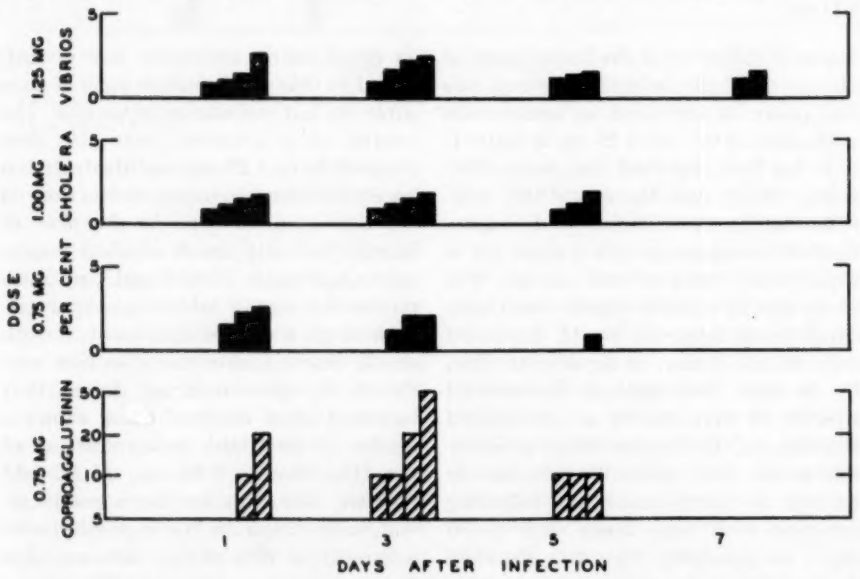


FIG. 3.—Infection of actively immunized animals 14 days after the last of 3 doses of cholera O vaccine. Coproantibody titer has declined to a low level. The ID_{50} dose is 0.70 mg.

ulation of antiserum, no immunity was detectable. Titration was carried out over the range 0.075 to 2.00 mg and the ID_{50} dose found to be 0.09 mg, not significantly different from that of the normal animal.

Antibody of heterologous origin may be introduced more directly into the lumen of the bowel by intragastric inoculation. It has been shown elsewhere (Burrows and Havens, 1948) that a portion of such antibody, possibly 25%, passes through the bowel and is excreted in the feces while, of the remainder, some is absorbed without loss of activity, appears in the serum in 10 to 14 days, and is at least in part secreted into the bowel in that it may be found in the feces. In these experiments the animals were given 2 ml of hyperimmune rabbit serum by the intragastric route 24 hours before infection to give significant coproagglutinin titers in most of the animals, 5 of the 6 in the group illustrated in figure 5. The results of titration over the dose range of 1.0 to 3.0 mg are shown there. The ID_{50} dose was calculated to be 0.36 mg, a 4-fold increase over the normal and indicative of an immunity somewhat superior to that resulting from parenteral antiserum, but still

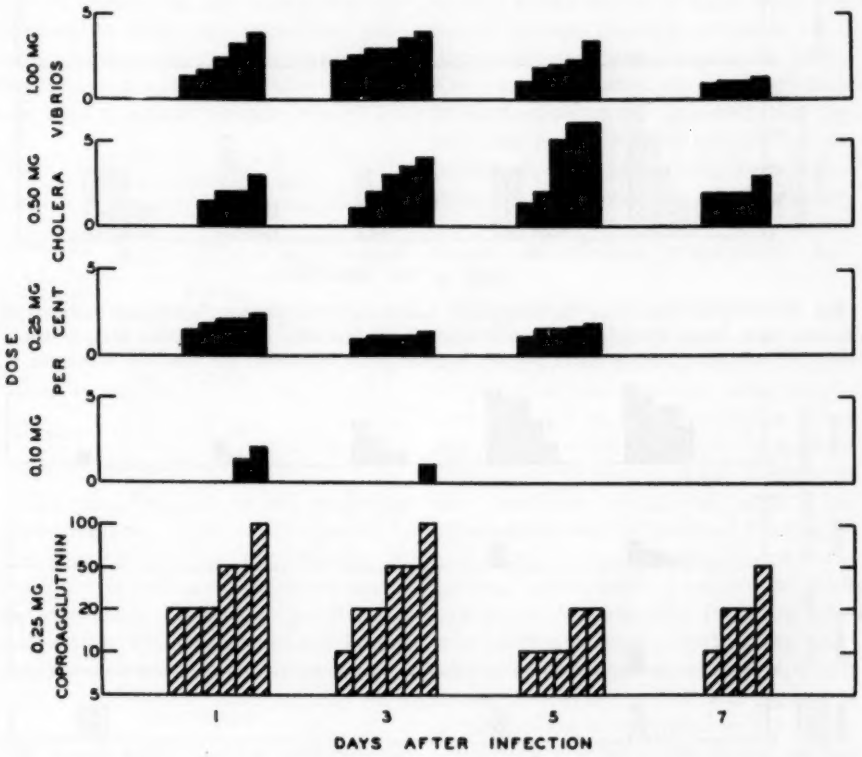


FIG. 4.—Infection of animals 1 day after passive immunization by the intraperitoneal route. Coproantibody is present to good titer, but the inefficacy of passive immunization is illustrated by comparison with the infection following a dose of 1 mg with that produced by the same dose in figures 2 and 3. The ID_{50} is 0.20 mg.

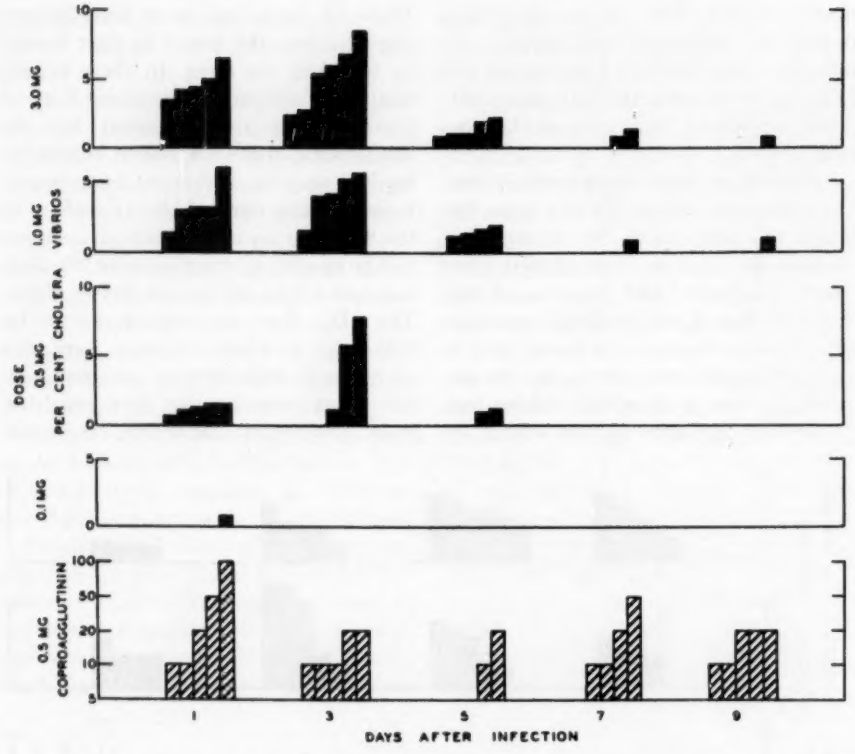


FIG. 5.—Infection 1 day after the intragastric inoculation of antiserum. Appreciable amounts of antibody have passed through the intestinal tract on the first day, and subsequent titers probably represent, at least in part, an active immune response to the infections. The ID_{50} dose is 0.36 mg.

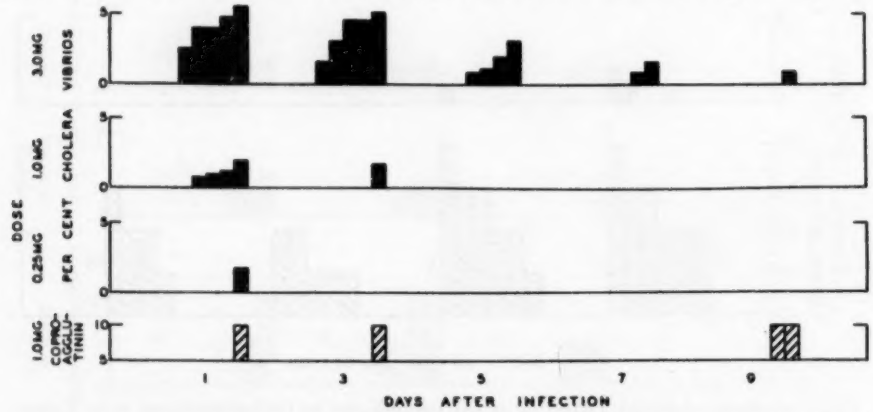


FIG. 6.—Infection 14 days after intragastric inoculation of antiserum. The increased immunity is apparent in the comparison of the amount of infection resulting from a 1 mg dose here with that in figure 5. The ID_{50} dose is 0.51 mg.

markedly inferior to active immunity.

A second titration was carried out in animals receiving intragastric antiserum 14 days earlier. By this time antibody was demonstrable in the serum and in small amounts in the feces. The latter is presumably antibody originally absorbed and then secreted into the bowel. This titration was carried out over the dose range 0.25 to 3.00 mg and the results are shown in figure 6. The ID_{50} dose was found to be 0.51 mg, representing a 6.4-fold increase over the normal. It is noteworthy that this is significantly larger than that found immediately after intragastric inoculation of antiserum and compares not unfavorably with the immunity persisting 14 days after active immunization, having approximately three-fourths of the fold increase.

TABLE 1.—*Prophylactic immunity as measured by fold increase in ID_{50} dose.*

Immunity	Time of infection	ID_{50} (dry weight)	Fold increase over normal	Percent of maximum*
None (normal)		0.08 mg		
Active	4 days	1.13 mg	14.1	100.0
	14 days	0.70 mg	8.7	61.7
	28 days	0.15 mg	1.9	13.5
Passive parenteral	1 day	0.20 mg	2.5	17.7
	14 days	0.09 mg	0.1	7.1
Passive intragastric	1 day	0.36 mg	4.0	28.4
	14 days	0.51 mg	6.4	45.5

* Level of significance ($P = 0.01$) = 11.4%.

The data from all of the foregoing experiments are summarized in table 1. The interrelation of the degrees of immunity is indicated as percentage of the maximum obtained, i.e., that at 4 days after the last vaccine inoculation of actively immunized animals.

DISCUSSION

It seems clear that a prophylactic immunity, effective in preventing infection and measurable as fold increase in the ID_{50} dose, may be produced in the guinea pig by various methods of immunization. Of these, active immunization was by far the most effective, but was transient in that it declined by 40% in the 10 days following peak antibody response, and almost 90% by 28 days after immunization. Assay of the level of immunity by titration of the ID_{50} dose appeared to be a more reliable and more delicate method than that of alteration in the pattern of vibrio excretion following infection with overwhelming doses. Thus the latter method showed no difference in the levels of active immunity at peak antibody response and 10 days later, and gave no evidence of the residual trace of immunity persisting for 28 days, while the former allowed this differentiation on a reasonably accurate quantitative basis. The relative inefficiency of passive immunization was demonstrable by both methods, but the apparent bacteriostatic effect of antibody that dominates the picture in passive immunity was shown to be reflected as a minor degree of effective prophylactic immunity.

These results are consistent with the hypothesis that coproantibody plays an integral, and possibly decisive, part in effective immunity to this enteric infection, but the relationship is not a simple one with agglutinin titer. The lack of correlation is most obvious in the passively immunized animal in which there was an artificial flooding of the bowel with antibody, directly following intragastric inoculation with antiserum or indirectly following passive parenteral immunization. The effective antibody appears to be that which is secreted into the bowel as a normal process. This is suggested in the markedly higher level of immunity in the actively immunized animal, but most strikingly in the animal immunized by intragastric inoculation with antiserum in which the initial level of immunity

was low despite the presence of coproagglutinin in seemingly significant titer, but actually increased by more than 50% 14 days after inoculation, coincident with the secretion of antibody from the tissues that had been originally absorbed from the bowel (cf. Burrows and Havens, 1948). As pointed out elsewhere (Burrows, 1953), it is possible that the efficiency of antibody in immunity to the enteric infection is dependent upon the presence of complement. In experiments to be reported subsequently the writer has observed that the amount of hyperimmune serum required to produce demonstrable lysis in vitro in the presence of complement is very small, 0.0001 ml in a total volume of 5 ml, and dilutions giving vibriolysis would not necessarily show an agglutinin titer of 1:10 or higher. Coproantibody could thus be present in effective titer and yet not be demonstrable as agglutinin. This degree of activity of antibody is consistent with the occurrence of a substantial degree of prophylactic immunity together with low agglutinin titer as at 14 days following active immunization, and with the trace of immunity detectable at 28 days in the absence of significant agglutinin titer.

The relative importance of pre-existing coproantibody and an anamnestic response in the immunity demonstrable subsequent to active immunization is uncertain. Though it may be sufficient to affect appreciably the level of immunity determined by titration of the ID_{50} dose, there was little indication in the agglutinin response to the test infection that it was of a high order.

The temptation to draw analogies between an experimental infection such as that reported here and human disease is a strong one. While their value is often uncertain, a number of resemblances may be noted here. Thus, the bacteriological character of the infection produced by minimal doses is not altered to an appreciable extent by active immunization, and this is consistent with the failure of immunization to modify the human infection. Similarly, the limited immunity conferred by passive immunization, and its failure to affect subsequent infection, have their counterparts in the therapeutic inadequacy of antiserum in human cholera.

In addition, the duration of prophylactic immunity in the guinea pig and in man is not dissimilar. The time relationship in their coproantibody response suggests that the rise and fall in immune response is approximately 4 times as rapid in the guinea pig as in man. Thus coproagglutinin begins to appear by the 2nd day following primary inoculation in the guinea pig, peak titer is reached in 4 to 6 days, and significant titer persists for 3 to 4 weeks following multiple inoculation. In man coproagglutinin is detectable 1 week after primary inoculation, reaches a peak in 4 to 5 weeks, and persists for 3 to 4 months after immunization. In the experiments reported here the level of prophylactic immunity in the guinea pig corresponded approximately to coproagglutinin titer following active immunization. It is generally said that immunity produced in man by inoculation with cholera vaccine appears very early and is detectable in some persons at the end of 1 week, and is of relatively short duration, possibly 6 months. Using a factor of 4, presumably detectable traces of immunity would remain at 4 months, which is not inconsistent with field observations. The implied function of coproantibody in prophylactic immunity to human cholera has not, of course, been demonstrated.

Comparison of prophylactic immunity in the guinea pig and in man may also be made in another way. Rarely

is naturally occurring disease so prevalent as to be present in 50% of the population at risk, though actual prevalence is no doubt greater than that indicated by morbidity rates that fail to take into account the asymptomatic infections that Frost has so vividly characterized as "occurring beneath the surface of our observation." Nevertheless, experiments based on the prevention of infection in a portion of animals inoculated by the intragastric route with borderline doses would seem to be not too far removed from natural conditions of infection in human cholera. The question of the relationship between morbidity rates of descriptive epidemiology and dosage is noteworthy here because of the coincidence of the 14-fold differential in cholera morbidity between immunized and nonimmunized groups noted in the Madras studies of 1942–43 (Adiseshan, Pandit and Venkatraman, 1947) and the 14-fold increase in ID_{50} dose resulting from active immunization in these experiments. If it is assumed that immunity, expressed as infection rate, is normally distributed, the degree of immunity found in man corresponds to that in which the ID_{50} dose for the guinea pig is 0.11 mg, just at the level of significance ($P=0.01$). The conclusion that the immunity described here is far superior to that observed in the Madras studies is, however, hardly justified. For example, the level of immunity characterized by a 14-fold increase in ID_{50} dose in the guinea pig was that observed at the height of the immune response, and it is quite improbable that all individuals immunized in the field studies noted above were infected at a corresponding peak in immunity. If the full level of immunity obtained here occurs in man, the observed differential in morbidity is consistent with a ratio of infections to cases of disease of about 10:1, i.e., the fold increase in the dose of 0.11 mg is 1.375. Or, if the average level of immunity at the time of infection corresponded to that observed in the guinea pig at 14 days, and possibly equivalent to that at 2 months in man, the ratio would become 5:1. The extent to which the casual carrier state or asymptomatic infection occurs in epidemic cholera is unknown and cannot be checked against such hypothetical ratios. In general, while it is clear that the experimental data may not be inconsistent with the results of field tests of prophylactic immunity in man, too little is known to allow more than a theoretical comparison.

CONCLUSIONS

1. A minimal dose of cholera vibrios given by the intragastric route that will infect 50% of guinea pigs may be determined and is designated the ID_{50} dose.

2. Prophylactic immunity to the enteric infection may be measured by fold increase in the ID_{50} dose in immunized animals over that for normal animals.

3. Active immunization with cholera O vaccine results in a 14-fold increase in the ID_{50} dose at the height of the immune response, 4 days after a course of vaccine, which declines to 8.7-fold at 14 days and 1.9-fold at 28 days.

4. Passive parenteral immunization produces an immediate immunity at a low level, with a 2.5-fold increase in ID_{50} dose, and no detectable immunity remains 14 days later.

5. Passive immunization by the intragastric route gives an immediate immunity with a 4-fold increase in ID_{50} dose that rises to a 6.4-fold increase at 14 days, coincident with the secretion of antibody from the tissues originally absorbed from the bowel.

6. Prophylactic immunity, like im-

munity to infection with overwhelming doses, is associated with the active secretion of antibody into the bowel, and it is suggested that coproantibody plays an integral and significant part in prophylactic immunity to this enteric infection. Its efficacy may be associated with the presence of complement.

REFERENCES

Adiseshan, R., Pandit, C. G. and Venkatraman, K. V. 1947, Statistical evaluation of anti-cholera inoculation as a personal prophylactic against cholera and its efficacy in the prevention and control of cholera epidemics. Indian J. Med. Res. **35**: 131–152.

Burrows, W. 1948, Excretion of antibody in feces and urine and its absorption from the bowel. Fed. Proc. **7**: 302.

———. 1953, Studies on immunity to Asiatic cholera. VI. Observations on the relation of antibody to effective immunity to experimental enteric cholera, with a note on sulfonamide therapy. J. Infect. Dis. **92**: 152–163.

Burrows, W., Elliott, M. E. and Havens, I. 1947, Studies on immunity to Asiatic cholera. IV. The excretion of coproantibody in experimental enteric cholera in the guinea pig. J. Infect. Dis. **81**: 261–281.

Burrows, W. and Havens, I. 1948, Studies on immunity to Asiatic cholera. V. The absorption of immune globulin from the bowel and its excretion in the urine and feces of experimental animals and human volunteers. J. Infect. Dis. **82**: 231–250.

Burrows, W., Mather, A. N., Elliott, M. E. and Havens, I. 1947, Studies on immunity to Asiatic cholera. III. The mouse protection test. J. Infect. Dis. **81**: 157–174.

Burrows, W. and Ware, L. 1949, Prophylactic immunity to experimental enteric cholera. Fed. Proc. **8**: 401.

STUDIES ON INTRACEREBRAL INOCULATION OF NEWCASTLE DISEASE VIRUS (NDV) INTO MICE

I. RESPONSE OF WEANLING MICE TO 25 STRAINS OF NDV

E. UPTON,* R. P. HANSON,* D. DOW,* AND C. A. BRANDLY†

From the Department of Veterinary Science, University of Wisconsin, Madison, Wisconsin

Newcastle disease virus (NDV) inoculated into the brain of mammals in various studies has produced death in some instances and has been without perceptible effect in others. In an effort to clarify the cause of this difference the activity of 25 selected strains of NDV was studied by intracerebral inoculation of weanling mice.

Henle and Henle[1,2] and Hale and McKee[3] observed a severe nervous reaction following intracerebral inoculation of influenza A and B viruses into 3- to 5-week-old mice. The effect was considered a toxic one since evidence of proliferation of the virus in the brain was not obtained. Henle and Henle[4] also demonstrated that influenza virus produced severe effects in mice when inoculated by the intravenous or intraperitoneal routes.

Since then other investigators have demonstrated that injection of a large quantity of certain viruses of the influenza-NDV group by various routes can elicit certain other disturbances in mammalian hosts.[5–8] The relationship of the neurotoxic effects of influenza and Newcastle disease viruses to factors producing other effects, such as febrile reactions, leucopenia, and drop in blood pressure, has not been clarified. In several instances, however, the neurotoxic agent was apparently more labile than these factors.[4,9–12]

The property of NDV which incites signs of central nervous system disturbance, without evidence of virus proliferation, will be designated the neuropathic factor (NF). This term is used to avoid implication that the reaction must be caused by a toxic part or toxic product of the virus.

Kraneveld and Nasoetion,[13,14] using chicken brain suspensions, were not able to induce nervous signs in mice by intracerebral inoculation of any of 50 Dutch East Indies strains of NDV, but several workers have reported the presence of a factor which was neuropathic for mammalian hosts when using infected allanto-amniotic fluids of other

Received for publication September 11, 1952.

Published with the approval of the Director of the Wisconsin Experimental Station.

* Department of Veterinary Science.

† Departments of Veterinary Science and Bacteriology.

1. Henle, G. and Henle, W. 1944, Science, **100**: 410–411.
2. Henle, W. and Henle, G. 1946, J. Exper. Med. **84**: 623–637.
3. Hale, W. M. and McKee, A. P. 1945, Proc. Soc. Exper. Biol. & Med. **59**: 81–84.
4. Henle, W. and Henle, G. 1946, J. Exper. Med. **84**: 639–660.
5. Chang, H. T. and Kempf, J. E. 1950, J. Immunol. **65**: 75–84.
6. Evans, C. A. and Rickard, E. R. 1945, Proc. Soc. Exper. Biol. & Med. **58**: 73–74.
7. Salk, J. E. 1948, J. Immunol. **58**: 369–395.
8. Sugg, J. Y. 1949, J. Bact. **57**: 399–403.
9. Wagner, R. R., Bennet, I. L., Jr. and LeQuire, V. S. 1949, J. Exper. Med. **90**: 321–334.
10. Bruyn, H. B., Meiklejohn, G. and Brainerd, H. 1949, Am. J. Dis. Child. **77**: 149–163.
11. Kempf, J. E. and Chang, H. T. 1949, Proc. Soc. Exper. Biol. & Med. **72**: 272–275.
12. Salk, J. E., Pearson, H. E., Brown, P. M., Smith, C. J. and Francis, T., Jr. 1945, Am. J. Hyg. **42**: 307–322.
13. Kraneveld, F. C. and Nasoetion, A. 1938, Nederland Ind. Bladen Dier. **50**: 356–361.
14. Kraneveld, F. C. and Nasoetion, A. 1941, Nederland Ind. Bladen Dier. **53**: 148–155.

strains of NDV. Strains 11914 and Herts of NDV, when administered intracerebrally by Brandly and coworkers,[15] often caused paralysis and death of weanling mice. Neither group of workers was able to adapt the virus of NDV to serial passage in weanling or older mice although the virus persisted in the brain as long as 10 days after inoculation.

Brueckner and coworkers[16,17] reported, in preliminary studies, the appearance of neuropathic signs in a variety of mammals following intracerebral inoculation of strain 11914 or a hamster-adapted line of 11914. The animals included cave bats, ferrets, rabbits, calves, sheep, mice, monkeys, and hamsters. Successful serial passages of 11914 were recorded with cave bats and hamsters. Cave bats, according to Reagan,[17] succumbed rapidly to strains of NDV from Colorado, Delaware, Kentucky, Minnesota and Connecticut. Reagan and coworkers[16,18] have reported serial passage of the hamster-adapted line of 11914 in rhesus monkeys and weanling mice. Kilham[19] serially passaged the Victorian strain of NDV in adult mice after initial adaptation to sucklings.

Wenner and coworkers[20] found that strain 11914 produced severe neuropathic signs in rhesus monkeys. Strain Manhattan-Kansas caused milder signs in monkeys but was innocuous for weanling mice.

Severe neuropathic responses by young pigs and sheep of several ages have been recorded by Hofstad,[21] who administered strain 125-Iowa intracerebrally. Attempts to adapt this strain of virus by intracranial passage in sheep and swine were unsuccessful.

MATERIALS AND METHODS

The 25 NDV strains, including 2 strain variants, were supplied by the NDV repository at the University of Wisconsin of the North Central Regional Technical Committee on Newcastle disease and the U. S. Bureau of Animal Industry. The strains originated from western, central and eastern sections of the United States as well as from several foreign countries. All strains were of avian origin, with the exception of 3, which were isolated from human eye infections. Two strains had been subjected to mammalian brain passage after recovery from chickens. The origin and subsequent history of each of the 25 viruses employed in these studies is summarized in table 1. Strains whose characteristics had been modified by prolonged egg passage or mammalian passage are designated as variants.

To provide freshly harvested fluids, 10-day embryonating eggs were inoculated into the allantoic chamber with 0.1 ml of a 10^{-2} dilution of virus. To circumvent bacterial contamination, since use of fluids was required before bacteriological examination could be made, 500 Oxford units of penicillin were injected with the inoculum for each egg. Fluids from eggs inoculated with penicillin alone and harvested after 2 days further incubation did not produce any reaction in mice inoculated intracerebrally. Fluids for inoculum were harvested as soon as possible after death of the embryo and kept at room temperature until used, since preliminary experiments had indicated that cold may alter the neuropathic properties of the virus. In almost all instances, less than 6 hours elapsed between harvest of the fluids and the inoculation of mice. Clear, undiluted, infected allantoic fluid from a single egg served as inoculum for a group of mice. For purposes of maintaining a comparative inoculum titer among strains of virus, only

15. Brandly, C. A., Moses, H. E., Jungherr, E. L. and Jones, E. E. 1946, Am. J. Vet. Res. **7**: 289–306.
16. Breuckner, A. L., Reagan, R. L., Schenk, D. M., Werner, H. O. and Hickman, J. N. 1950, Proc. Book Am. Vet. Med. Assoc. 163–165.
17. Reagan, R. L., Smith, E. J. and Breuckner, A. L. 1950, Proc. Soc. Exper. Biol. & Med. **75**: 691–692.
18. Reagan, R. L., Werner, H. O. and Breuckner, A. L. 1950, J. Infect. Dis. **87**: 210–212.
19. Kilham, L., Loomis, L. N. and Peers, J. H. 1952, Am. J. Vet. Res. **46**: 95–98.
20. Wenner, H. A., Monley, A. and Todd, R. W. 1950, J. Immunol. **64**: 305–322.
21. Hofstad, M. S. 1950, Cornell Vet. **40**: 191–198.

TABLE 1.—*Origin and passage history of 25 NDV strains used in experiments on neuropathogenicity*

Strain and place of origin	Source of isolation	Subsequent passages
1. *Hertfordshire*—England E_8	Chicken	$C_{10}E_8$
2. *Hertfordshire*—England E_{61}	Chicken	$C_{10}E_{61}$
3. *Milano*—Italy	Chicken	E_9
4. *Ixtapalapa*—Mexico	Chicken	E_7
5. *Texcoco*—Mexico	Chicken	E_6
6. *Victoria*—Australia	Chicken	E_{5UW}
7. (T) *Peerless*—Saskatchewan	Turkey	*E_{5UW}
8. *Labelle*—Ontario	Chicken	*E_{5UW}
9. *Cleroux*—Ontario	Chicken	*E_{7UW}
10. *Watford*—Ontario	Chicken	*E_{9UW}
11. *11914*—California—*FD*	Chicken	E_{11}
12. *11914—Reagan*	Chicken	$H_{20}E_5H_1E_1H_1E_{5UW}$
13. *RO*—California	Chicken	E_{5UW}
14. *CG 179*—California	Chicken	$E_{175_{UC}}E_{10UW}$
15. *125—Iowa*	Chicken	Alter. mammal. chick embr. pass., E_{5UW}
16. *23—Iowa*	Chicken	E_{10}
17. (H) *Najarian*—Ohio	Human eye infection	E_{5UW}
18. (H) *Miller*—Ohio	Human eye infection	E_5
19. (H) *Lee*—Missouri	Human eye infection	E_{7UW}
20. *GB*—Texas	Chicken	E_{10}
21. *Roakin*—New Jersey	Chicken	E_{14}
22. *KD*—New Jersey	Chicken	E_{15}
23. *46967*—Michigan	Chicken	E_{15}
24. *BI*—Virginia	Chicken	E_{17}
25. *Manhattan—Kansas*	Chicken	E_{19}

C—chicken passages.
E—egg passages.
E_{UC}—egg passages at Univ. of California.
E_{UW}—egg passages at Univ. of Wisconsin.
H—Hamster passages.
* Previous egg passages are known to be few.

allantoic fluids were employed, since Hanson[22] has shown that certain strains of NDV produce considerably less virus, as indicated by the hemagglutination titer, in the amniotic than in the allantoic fluid of the embryonating egg. All inoculums were tested for bacterial sterility and the presence of hemagglutinins.

The Swiss albino mice employed in these studies were from a line originating at the Rockefeller Institute and had been obtained from Iowa State College for developing a stock colony at the Veterinary Science Department. The majority of mice were between 21 and 30 days old although in a few instances mice as old as 50 days were used. Each test group consisted of 6 to 9 mice. Eight serial intracerebral passages of normal mouse brain suspensions in mice did not reveal the presence of any latent virus which might be activated by inoculation procedures.

The mice were anesthetized with ether before injection. A 26-gauge needle and a 0.25-ml tuberculin syringe were used for the inoculation. Five-hundredths ml of infected allantoic fluid was injected into the right cerebral hemisphere of each mouse. Tests were repeated at least once with each virus. The animals were observed over the subsequent 11-day period. Control mice received normal allantoic fluids.

To facilitate comparison of potency of strains a "neuropathic index" was devised. Values were derived for the separate test groups on the basis of the severity of signs manifested by each animal in the group. The highest score, 4, indicates that the animal was completely prostrate or severely paralyzed, or both, or was found dead. When a mouse showed evidence of paralysis or other severe nervous involvement but did not become prostrate or did not die within the 11-day period, it was given a value of 3. The number 2 indicates that only transitory lameness or other mild nervous signs, such as temporary hyperirritability and incoordination, were observed. Such mice also had marked anorexia, or ruffled hair coat, or definite sluggishness, or a combination of all of the various signs. The presence of very mild signs such as detectable sluggishness, inappetance or incoordination is indicated by the number 1. A complete absence of clinical signs is expressed as 0.

The sum of the scores for all affected mice in a group is divided by the total number of mice in the group. For example, in a group where 4 of the 8 mice died, 2 showed a definite stiffness of the legs and 2 remained normal, the neuropathic index for the strain of virus is calculated as follows:

$$\frac{(4\times4)+(2\times2)+(2\times0)}{8}=\frac{20}{8}=2.5$$

It is true that this neuropathic index (NI) permits only rough comparisons and that an inadequate evaluation may be obtained especially

22. Hanson, R. P. 1949, Thesis, Univ. of Wisconsin, Madison, Wis.

in the instances where only one or two mice of a group show severe signs.

RESULTS

The neuropathic indices of each of 25 strains of NDV are compiled in table 2. The indices demonstrate that 8 of the 25 strains had the ability to cause death in a high proportion of the mice injected. A large percentage of mice inoculated with strains 11914-Reagan, Milano, 125-Iowa, CG 179 and RO were prostrate or dead within 16 or 20 hours after inoculation. Often the first sign of central nervous system involvement was marked hyperirritability which might be noticeable as early as 3 to 4 hours after injection. The condition of the animal progressed rapidly toward prostration and death, which usually followed within 16 to 48 hours after injection. Where the central nervous system disturbances progressed more slowly, typical spastic paralysis of the hind limbs or both front and hind limbs preceded death on the second day. Clonic spasms were of rather frequent occurrence. Twitching, tremor and other manifestations of severe nervous disturbance were common also. Mice receiving the lethal strains often developed rough hair coats and sluggishness and were otherwise in general ill-health prior to development of the severe nervous manifestations.

TABLE 2.—*Relationship of the neuropathic index of NDV strains to their chicken LD_{50} and hemagglutination and embryo infectivity titers.*

Strain	HA titer, allantoic fluid	Log embryo LD_{50}	Log chicken LD_{50}	Neuropathic index
	40	7		4.0
1. CG 179	1280	9	7	4.0
2. Milano	1280	9	8	4.0
3. RO	1280	9	8	4.0
4. 11914-FD	1280	9	7	4.0
5. 11914-Reagan	80	7	5	4.0
6. GB	1280	9	7	3.7
7. 125-Iowa	1280	9	5	3.6
8. Miller	1280	9	4	3.3
9. Texco	1280	9	1	2.3
10. MK	1280	9	2	2.3
11. Herts-E_8	1280	9	8	2.2
12. Ixta	1280	9	1	2.0
13. Cle	1280	9	4	2.0
14. 23-Iowa	1280	8	2	1.4
15. Herts-E_{62}	1280	9	4	1.2
16. Lab	1280	9	2	1.0
17. B1	1280	9	0	0.8
18. Roakin	1280	9	0	0.6
19. Peer	1280	9	0	0.6
20. KD	1280	9	0	0.5
21. Len	1280	9	1	0.5
22. 46967	1280	9	0	0.3
23. WAT	1280	8	2	0.0
24. Naj	1280	9	0	0.0
25. Vict	1280	8		1.9

In only a small portion of the mice injected did any of the strains—Ixta, MK, Texco, Herts-E_8, and Cle, induce as severe neuropathic signs as those invoked by the 8 lethal strains. Nevertheless, all of the 5 strains produced in every mouse inoculated signs of marked anorexia, ruffled hair coat, slight to moderate stiffness and lameness or transient incoordination. These symptoms usually developed within 48 hours after injection. Hyperirritability of milder nature than that exhibited by animals receiving the highly lethal strains has been observed as early as 3 to 4 hours post injection. The more severe signs were delayed, at times, beyond the second day.

Eleven of the remaining strains, Vict, 23-Iowa, Herts-E_{62}, Lab, B1, Wat, KD, Len, 46967, Peer and Roakin, produced signs only irregularly, and these were usually of an extremely mild and transient nature. The severity of the central nervous system involvement engendered was usually of a very low order. However, with almost every strain, an occasional mouse of a group became paralyzed and died. The period before appearance of severe signs in these few animals was often delayed until 6 to 8

days after injection. Strain Naj was the only one studied that failed to produce any severe nervous effects in the weanling mouse. Nevertheless, even this strain has caused a definite but transitory lameness in a few mice of control groups in other experiments.

In view of the uniformly high hemagglutination and embryo infectivity titers of infected fluids used as inoculum, it seems unlikely that the results obtained can be explained on the basis of quantitative differences among strains. From table 2 the usual hemagglutinin and embryo infectivity titers of individual strains may be compared with representative neuropathic indices. The embryo infectivity and hemagglutination titers are usually high regardless of the NI of the strain. The few instances where the hemagglutination titer is low occur in strains lethal for mice.

The majority of replicates gave results essentially the same as those obtained in the original tests. In some instances, however, the neuropathogenicitity was of a lower order than was usual for the strain. The low indices, unexplainable at present, are not related to the hemagglutinin or egg infectivity titer of the fluid (unpublished data). Strain CG was the most erratic in behavior. This strain has varied greatly also in regard to its hemagglutinating activity and embryo infectivity.[23] Control mice receiving uninfected allantoic fluids did not develop perceptible reaction on intracerebral inoculation.

Efforts have been made to determine possible correlations between the degree of neuropathogenicity with the ability of strains of NDV to agglutinate red cells of various mammalian species, to produce hemagglutinins in the amniotic fluids of embryonating chicken eggs, to induce pneumopathic effects in mice and to be pathogenic for chickens. Other properties also compared with the neuropathic property were the persistence of embryo infectivity and hemagglutinating activity at 56 C. Studies of these properties other than neuropathogenicity have been described by Hanson.[22*]

In most cases the various characteristics were not correlated, but a significant relationship between the chicken LD_{50} and the ability to cause a nervous response in mice was observed among strains. As a rule, the strains most lethal for chickens by the intravenous route had the highest neuropathic index. Correlation of these characteristics is apparent from the data depicted in table 2 and figure 1. Four strains, Miller, MK, Ixta and Texco, for which tests have not been made in mice and chickens with fluids of the same egg passage were not included in figure 1. The extreme importance of using virus of the same egg passage in both species was demonstrated when it was found that a line of GB which on egg passage had lost its pathogenicity for chickens had also lost much of its neuropathogenicity for mice. Fluid from the original stock which was lethal to chickens also killed mice. Similar findings have been made with different lines of 11914 (unpublished data). A line of 11914 designated BAI 15 differed from the line 11914 FD used here in that the pathogenicity for chickens and neuropathogenicity for mice of the BAI 15 line were considerably lower than that of the FD line. Strain Herts E_8 and its variant, E_{62}, were consistently more

23. Bang, F. B. 1950, Personal communication.

* Strains 125—Iowa, Reagan, Naj and Len have been tested for chicken lethality since the completion of Hanson's work. Repeat titrations have been run on certain strains recorded in the above report. In all cases the strains have been titered by intravenous inoculation of 6- to 10-week-old chickens.

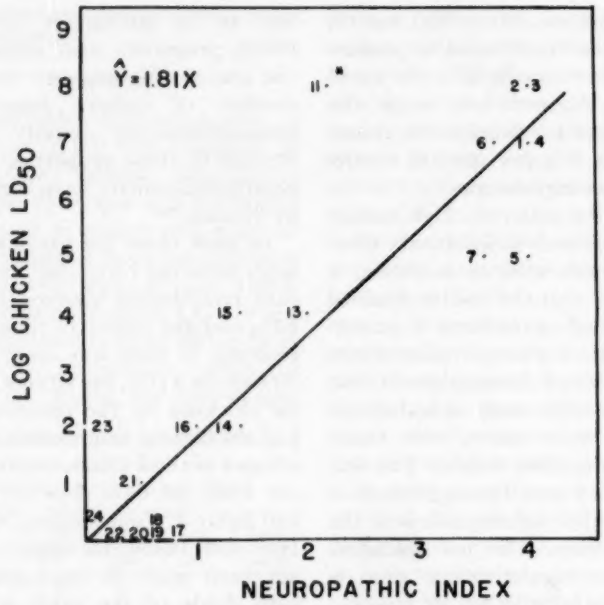


Fig. 1—Relationship of mouse neuropathic indices of 20 strains of NDV to their pathogenicitity for chickens.

* Numbers correspond to number of the strain in table 2.

lethal for birds than for inoculated mice. The mammalian passaged lines 11914-Reagan and 125-Iowa were more lethal for mice than for chickens. It appears that there is generally a good correlation between mouse lethality from intracerebral inoculation and chicken pathogenicity from intravenous inoculation, especially when fluids of the same egg passage are used in both tests.

A possibly significant relationship between the "allanto-amniotic index"[22] and the neuropathic index also exists. Those strains having a high allanto-amniotic index, i.e., those whose amniotic fluids had high hemagglutination titers, tended to have low neuropathic indices although this was not invariably the case.

DISCUSSION

While certain strains of NDV possess the ability to cause a severe neuropathic reaction in intracerebrally injected mice and others possess this capacity to a lesser degree, it seems quite likely that all strains have some ability to damage specifically the central nervous system under certain conditions. The nature of the host-virus interaction which results in the clinical manifestation of central nervous system disturbance as a result of intracerebral inoculation with the majority of NDV strains remains to be elucidated.

A delay of 3 to 8 days in the appearance of nervous signs in some weanling mice receiving the less neuropathic strains suggests that NDV may have potential ability to multiply as in a true infection, this ability being masked in highly neuropathic strains. The period of latency suggests a growth cycle process of the virus. The possible relationship of certain stages of the infectious process of NDV and influenza

virus to their production of toxic reactions in mice has been investigated. Thus, Wagner[24] discovered that the neurotoxic action of influenza could be prevented by prior intracerebral injections of sublethal amounts of influenza virus or of cholera filtrate. The effect was considered to be due in part to destruction of susceptible cell receptors. In studies of pneumotoxicity of 2 strains of NDV in mice, Ginsberg[25] presented evidence that union of virus and susceptible cell must occur before formation of toxic lesions although there was no viral multiplication. Both receptor destroying enzyme and influenza virus prevented development of a toxic reaction.

Schlesinger[26] suggested that the neurotoxic signs induced by influenza virus in the brains of mice receiving non-neurotropic strains of the agent may be the result of a partial growth process. The uncomplete cycle consisted of a temporary rise in hemagglutinin and complement-fixing antigens without a similar rise in the infective titer.

Cairns[27] has reported that the infectivity titers of a strain of NDV and non-neurotropic influenza virus undergo a brief although temporary rise within a few hours after inoculation into mice. This phenomenon corresponds to a one or two step growth cycle.

Such reports have established that when certain strains of influenza virus and NDV are inoculated into mice, proliferation does not take place, but there is absorption of virus and initiation of an aberrant growth process. These events apparently are independent of the ability of the virus to incite disturbance of the nervous system. Wagner,[24] Cairns[27] and Schlesinger[26] worked with neurotoxic strains of influenza. The highly pneumotoxic Hickman strain of NDV was used by Ginsberg in experiments with interference by influenza and by receptor destroying enzyme. Cairns[27] used the Victoria strain of NDV, which in our laboratory has low neuropathogenicity.

Ginsberg[25] considers the infectivity and manifestation of pneumotoxicity to be independent. Lung lesions caused by NDV continued to increase as the infectious titer decreased.

It is known that living or non-living substances may act as lethal or as mildly injurious factors, depending on the degree of antagonism which they incite within the host animal. Henle and Henle[28] suggested the possibility that in the general adaptation syndrome of Selye the toxicity of influenza virus could be due to the "alarm reaction" of the animal body to an adverse situation. The NDV particle may have a property which is apart from its infective ability and is capable of profoundly disturbing the normal functions of certain cells of the brain. This property may be present either in different quantities or it may differ in nature among strains, the amounts of virus absorbed being the same in both toxic and innocuous strains.

The less actively neuropathic strains may engender less of the "neurotoxic factor," with a resultant increase in latency, as in the case of diluted exotoxins.[29,30] Conversely, the more lethal

24. Wagner, R. R. 1952, Brit. J. Exper. Path. **33**: 157–167.
25. Ginsberg, H. S. 1951, J. Exper. Med. **94**: 191–211.
26. Schlesinger, R. W. 1950, Proc. Soc. Exper. Biol. & Med. **75**: 541–548.
27. Cairns, H. J. F. 1951, Brit. J. Exper. Path. **32**: 110–117.
28. Henle, W. and Henle, G. 1947, J. Exper. Med. **85**: 347–364.
29. Jordan, E. O. and Burrows, W. 1945, Textbook of Bacteriology, Philadelphia, W. B. Saunders Co.
30. Moll, T. and Brandly, C. A. 1951, Am. J. Vet. Res. **12**: 355–363.

strains may resemble concentrated toxins in rapidity of action because of a greater concentration of the neuropathic factor.

In support of the view that the lethality of NDV strains is a property of the strain independent of its ability to proliferate, there is evidence that chicken pathogenicity is not solely a result of the proliferation of the virus in the avian nervous system. Virus may be found in the brain in fairly high titer and yet signs may be mild or absent. Sinha and coworkers[31] found that strain MK developed to a titer of 10^9 embryo LD_{50} in the brains of 6 of 12 inoculated birds; the 6 remaining birds recovered without sequelae. In contrast, strain CG was found irregularly and in low titer in the brain of 6 of 12 birds, while the 6 birds not sacrificed were killed by the virus. If lethality for birds depends in part upon properties other than the ability of the virus to proliferate in the central nervous system or elsewhere in the body, relationship between neuropathogenicity for mice and the lethality for chickens may be based on a specific neuropathic factor of the virus.

Experience with formalinized, concentrated influenza vaccine indicates that the virus-protein complex is itself rather toxic to man.[10,12] Other experiments with partially denatured influenza and Newcastle disease viruses show that fever and hypotension may be produced equally well by a complete virus particle or by one having lost some of its more labile characteristics.[9,11] It is possible that the action of NDV in the mouse brain is a consequence of the whole protein-complex of the virus.

31. Sinha, S. K., Hanson, R. P. and Brandly, C. A. To be published.

SUMMARY

1. Twenty five strains, including 2 strain variants of Newcastle disease virus, were examined for their ability to cause central nervous system disturbances in weanling mice inoculated intracerebrally. Eight of the strains produced a high percentage of fatalities in inoculated mice. Five strains were capable of eliciting severe nervous signs in a small proportion of injected mice and milder signs in a large proportion of mice. Eleven strains did not cause apparent signs in a large portion of mice injected; when signs developed they were usually very mild although paralysis and death occurred occasionally. One strain of virus only rarely affected weanling mice. From these results it can be concluded that strain variation is the probable cause of discrepancies between different reports of the neuropathic activity of Newcastle disease virus.

2. Strains of Newcastle disease virus that induced severe involvement of the central nervous system in weanling mice inoculated intracerebrally were, as a rule, highly lethal for chickens when inoculated intravenously.

3. The possible nature of mechanism of the neuropathic property of Newcastle disease virus for mice and its correlation with lethality for fowl are discussed.

HISTOCHEMICAL INVESTIGATION OF THE SPHERULE OF *COCCIDIOIDES IMMITIS* IN RELATION TO HOST REACTION

J. E. TARBET AND A. M. BRESLAU

From the Veterans Administration Center, Los Angeles 25, California and the Division of Dermatology, Department of Medicine, University of California Medical Center, Los Angeles 24, California

The spherules of *Coccidioides immitis* in histologic preparations of infected tissue vary greatly in size and structure. While these differences are no doubt due to the developmental stages of the fungus, the histopathologist examining any particular slide can readily sort these spherules into two quite distinct principal groups: (a) large, thick-walled spherules, including intact sporangia, and (b) small fungus cells, inclusive of sporangiospores. The spherules of the first group range in size from 8 to 80 μ in diameter, with rare individuals up to 262 μ.[1] Normal sporangiospores measure 2 to 3 μ[2] and may be included in the second group of small forms. Experimental studies and observations have established that the host's cellular response to isolated fungi of group *a* is generally mononuclear, while the reaction to the spherules of group *b* is polymorphonuclear in type.[2–5] When the lesion contains both large and small spherules in close proximity the histopathologic reaction is mixed.

Menkin[6] has pointed out the important influence of the biochemistry of the parasitic microorganism in modifying the host's inflammatory reaction. It has been the purpose of the study reported here to ascertain significant chemical differences between the two main groups of spherules through the utilization of some of the newer histochemical techniques, and to correlate such differences, if any, with the differential host response to the two principal types of spherules as noted above.

The stains employed include the Korson differential trichome technique for nucleic acids,[7] the Baker acid-hematin test for phospholipid,[8,9] and the Lison α-toluidine blue reaction for mucopolysaccharide.[10] The safranin-fast green technique commonly used to stain plant materials[11] was also used to

Received for publication September 11, 1952.

The authors gratefully acknowledge the assistance given by William L. Martinsen in the preparation of the photomicrographs.

1. Baker, E. E., Mrak, E. M. and Smith, C. E. 1943, The morphology, taxonomy, and distribution of *Coccidioides immitis Rixford* and *Gilchrist 1896*. Farlowia, **1**: 199–244.
2. Tarbet, J. E., Wright, E. T. and Newcomer, V. D. 1952, Experimental coccidioidal granuloma; developmental stages of sporangia in mice, Am. J. Path. **28**: 901–917.
3. Baker, R. D. 1947, Tissue changes in fungous disease. Arch. Path. **44**: 459–466.
4. Forbus, W. D. 1949, Granulomatous Inflammation, Pub. no. 1, Am. Lect. Ser., Springfield, Ill., Chas. C Thomas.
5. Tager, M. and Liebow, A. A. 1942, Intranasal and intraperitoneal infection of the mouse with *Coccidioides immitis*. Yale J. Biol. & Med. **15**: 41–49.
6. Menkin, V. 1950, Newer Concepts of Inflammation, Pub. no. 70, Am. Lect. Ser., Springfield, Ill., Chas. C Thomas.
7. Korson, R. 1951, A differential stain for nucleic acids. Stain. Tech. **26**: 265–270.
8. Baker, J. R. 1946, The histochemical recognition of lipine. Quart. J. Micr. Sc. **87**: 441–470.
9. Baker, J. R. 1947, Further remarks on the histochemical recognition of lipine. Quart. J. Micr. Sc. **87**: 463–465.
10. Lison, L. 1936, Histochemie Animale, Paris, Gauthier-Villars. Cited by Glick.[21]
11. Johansen, D. A. 1940, Plant Microtechnique, New York, McGraw-Hill Co., Inc.

some advantage, in conjunction with digestion procedures for cellulose and chitin in an effort to obtain new information concerning the composition of the outer wall of the spherules.

The Korson differential trichome technique for nucleic acids was utilized as a means of distinguishing dormant spherules. It has been shown that activity of the ribonucleoproteins in the cell accompanies intense cytoplasmic synthesis[12] and that increase and concentration of the desoxyribonucleoproteins attends mitosis.[13] A diffuse, equivocal or weak test for nucleic acids can thus logically be employed as an indicator of dormancy in the cell. Dormant spherules could be expected to exert their influence on the host largely in the capacity of foreign bodies,[8] and the host reaction to static parasites might well be of a nature contrasting with the host response to other, actively metabolizing fungi.

The Baker acid-hematin test for phospholipid was utilized in another approach. The alternating chemotaxis of the inflammatory cells of the host, which correlates in general with the size of the fungi in the tissue, may hinge on the composition of the fungus wall at different growth stages. Cell coverings which resist the diffusion of large molecules presumably retain certain chemotactic substances within the cell, and block or alter chemical interchange between parasite and tissues. Lipoid complexes, particularly those rich in phospholipid, such as the lipoprotein of the stroma of erythrocytes, are evidently present in many selectively permeable biological structures.[14,15] It was thought that if one group of spherules possessed walls containing more phospholipid than another, this fact would assist in the formulation of an understanding of the alternating host response.

The Lison α-toluidine blue reaction for mucopolysaccharide was employed to locate this material in the spherules. The prosthetic groups of important microbial enzymes such as hyaluronidase may contain mucopolysaccharide.[16] Certain bacterial antigens are known to be mucopolysaccharide in nature (pneumococcus, Shiga-Kruse). Barnet,[17] Meyer[18] and others have presented evidence that the site of antigen-antibody activity in other nonbacterial cells also coincides with the location of materials giving positive tests for mucopolysaccharide. Menkin[6] has pointed out that inflammatory processes are essentially a manifestation of the immunologic mechanism. If mucopolysaccharides were found to be present in some spherules and not in others, the contrasting histopathologic response seen with different types of spherules might be interpreted with some justification as the reflection of enzymic or antigenic differences between them.

12. Casperson, T. and Schultz, J. 1940, Ribonucleic acids in both nucleus and cytoplasm and the function of the nucleolus. Proc. Nat. Acad. Sc. **26**: 507–515.
13. Brachet, J. 1950, Chemical Embryology, New York, Interscience Publishers, Inc. p. 447.
14. Dziemian, A. J. 1939, The lipid content and the permeability of the erythrocyte. J. Cell. & Comp. Physiol. **14**: 103–116.
15. Waugh, D. F. and Schmitt, F. O. 1940, Investigation of the thickness and ultrastructure of cellular membranes by the analytical leptoscope. Cold Spring Harbor Symposium, **8**: 233–241.
16. Palitz, L. L. and Brunner, M. J. 1950, The mucinoses: a classification with histochemical studies on the nature of mucin. J. Invest. Dermat. **14**: 159–172.
17. Barnet, F. M. 1951, Mucoproteins in relation to virus action. Physiol. Rev. **31**: 131–150.
18. Meyer, K. 1945, Mucoids and glycoproteins. Advances in Protein Chem. **2**: 249–275.

METHODS

Infection of mice with C. immitis.—An inoculum containing spherules of *C. immitis* was prepared by grinding infected tissue to small particle size in normal saline solution. Mature male ABC mice were injected intraperitoneally with 0.2 ml of the material. When signs of illness were seen in the mice (2 to 4 weeks postinoculation) they were killed. Grossly pathological specimens of liver, spleen and omentum were removed and placed in suitable fixative solutions.

Korson's differential trichrome technique[7] *for nucleic acids.**—Korson has found that after conditioning of the sections with orange G, a mixture of methyl green and toluidine blue colors ribonucleic acid (RNA) blue and desoxyribonucleic acid (DNA) green and that the reaction is probably semiquantitative. Photomicrographs taken with ultraviolet and visible light confirmed that structures containing nucleic acids were those which stained with the methyl green and toluidine blue. Digestion with nucleases and extraction with perchloric acid further indicated specificity for Korson's stain. In the employment of this technique, the present authors pretreated alternate sections of tissue containing *C. immitis* with perchloric acid prior to staining. The acid preferentially destroys RNA, unmasking the desoxy compound so that alternate slides located the two types of nucleic acid.

Baker's acid-hematin test[8,9] *for phospholipid.*†—The reaction of Baker's modification of the Smith-Dietrich acid-hematin test has been shown to be completely negative with cellulose and chitin[8] and is thus of special value in the staining of fungus cells. Phospholipid-containing material is mordanted with calcium chloride and potassium dichromate, enabling it to form a salt with borax hematoxylin. Extra-nuclear dark blue color locates the phospholipid in the sections. As a check upon the specificity of the reaction, alternate sections were extracted with a number of fat solvents prior to staining.

Lison[10] *stain.*‡—Michaelis[20] has shown that the

* Tissues were fixed overnight in Carnoy's fluid, washed in 95% ethanol for 1 hour. After clearing and infiltration with paraffin, they were sectioned and brought down to water. At this point alternate sections were treated with 10% perchloric acid at room temperature for 30 minutes. The perchloric acid-treated sections were then washed for 1 hour in running water and placed in a bath of distilled water for 10 minutes. All sections were then stained in 5% aqueous solution of orange G for 5 minutes and rinsed in distilled water. They were next stained in 0.15% aqueous solution of methyl green for 15 minutes. After draining, they were passed to distilled water containing 0.05% toluidine blue and 0.075% methyl green for 5 minutes. The stained slides were rinsed well in tertiary butyl alcohol and left overnight for differentiation in fresh tertiary butyl alcohol. They were cleared with xylene. The mounting agent was Permount (Fischer Scientific Company).

† Tissues were fixed for 72 hours in the following: formalin, 10 ml; 10% aqueous solution of calcium chloride, 10 ml; distilled water, 80 ml; powdered chalk, 2 g. Tissues were then incubated overnight at 60 C in an aqueous solution containing 3% potassium dichromate and 1% calcium chloride. They were washed in running water for 1 hour, followed by distilled water for 5 minutes, dehydration and infiltration with 5% gelatin in distilled water at 55 C over anhydrous calcium chloride. The infiltrated material was then frozen with carbon dioxide, sectioned and brought down to water. At this point alternate sections to be used as controls were bathed in 95% ethanol at 60 C for 1 hour to remove waxes. They were next treated with a mixture of equal parts of chlorophorm and ether for 1 hour to extract phosphatides, followed by immersion in acetone for 1 hour to dissolve glycerides and cholesterides. This procedure for extraction of lipids was suggested by Chargoff.[19] After acetone the treated alternate sections were washed in several changes of distilled water for 1 hour. All sections were then stained in Koltchinsky's hematoxylin at 37 C overnight. The following morning they were washed for 1 hour in several changes of tap water. Following an additional 10 minutes in distilled water, they were mounted in glycerin jelly.

19. Chargoff, E. 1944, Lipoproteins. Advances in Protein Chem. **1**: 1–25.

‡ Tissues were fixed overnight in equal parts of 8% basic lead acetate and 16% formalin. After washing for 1 hour in running water, the tissues were dehydrated, cleared and embedded in paraffin according to usual procedure. All sections were run down to water. Alternate sections were then bathed in 1% potassium hydroxide for 20 minutes at room temperature and washed for 30 minutes in running water. All sections were then stained in 0.1% toluidine blue in 1% ethanol for 1 hour. After thorough rinsing in 95% ethanol, they were blotted dry and placed in acetone for 5 minutes, followed by immersion in a mixture of equal parts of acetone and xylene for 5 minutes. Two subsequent changes of xylene for 3 to 5 minutes each were done for clearing. The sections were mounted in Permount.

α fraction of toluidine blue can be caused to act specifically with the sulfuric ester moiety of acid mucopolysaccharides, giving an alcohol resistant reddish color. The Lison stain is based upon the α-toluidine blue reaction after mordanting with basic lead acetate. As a check upon the specificity of the reaction, alternate sections were treated with potassium hydroxide before staining.

Cellulose and chitin.§—A number of generally used microchemical tests for the differentiation of chitin and cellulose, including Gilson's, Schulze's, Bethe's, Zander's and others[21,22] gave no satisfaction in the hands of the present authors. Results with them were not reproducible, and known positive and negative control materials stained in an equivocal or nonspecific manner. Another approach to the problem of chitin as against cellulose in the wall of *C. immitis* appeared to be required. This approach was found in the employment of the safranin-fast green combination[11] used to stain botanical specimens, in conjunction with digestions of the sections of *C. immitis* and of sections containing known chitin and cellulose. Privet leaf (cellulose) and eggs of ascaris (chitin) were utilized as the controls. Schweitzer's reagent was used to destroy cellulose and chlorine[23] to destroy chitin.

20. Michaelis, L. 1947, Nature of interaction of nucleic acids and nuclei with basic dyestuffs. Cold Spring Harbor Symposium, **12**: 131–142.

§ All tissues for the study of cellulose and chitin were fixed overnight in Carnoy's fluid and washed for 1 hour in 95% ethanol. After clearing and infiltration with paraffin, the specimens were sectioned and brought down to water. The first section of each specimen was stained in 0.5% safranin containing a few drops of formalin, 10 minutes at 60 C. The first sections were then washed in distilled water and differentiated in a saturated solution of picric acid in 95% ethanol for 15 seconds; this was followed by ammoniated 95% ethanol (10 drops of ammonium hydroxide in 100 ml 95% ethanol) for 15 seconds, to stop the action of the picric acid. The sections were then passed through two baths of absolute alcohol for 5 minutes each, then 0.5% fast green in equal parts of clove oil and isopropyl alcohol for 2 minutes, clove oil for 15 seconds, equal parts of clove oil, absolute alcohol and xylene for 15 seconds, and finally two changes of xylene until the sections cleared. Permount was used for mounting.

The second sections from each specimen were digested in a solution of copper carbonate in a minimum quantity of strong ammonia water (Schweitzer's reagent) for 1 hour prior to staining to remove cellulose. These sections were rinsed briefly in strong ammonia water and washed for 5 minutes in each of two changes of distilled water after the treatment with Schweitzer's reagent, and then subjected to the same staining procedures as the first sections.

The third sections from each specimen were placed in 5% sodium hypochlorite in 50% ethanol for 1 hour to destroy chitin, washed in running water for 1 hour, and for 5 minutes in distilled water, before staining.

21. Glick, D. 1949, Techniques of Histo- and Cytochemistry, New York, Interscience Publishers.
22. Rawlins, T. E. 1933, Phytopathological and Botanical Research Methods, New York, John Wiley and Sons, Inc.
23. Campbell, F. L. 1929, Detection and estimation of insect chitin and the irrelation of "chitinization" to hardness and pigmentation of the cutica of the American cockroach, *Periplaneta americana L.* Ann. Ent. Soc. Am. **22**: 401–426.

OBSERVATIONS

Distribution of ribonucleic acids (Korson stain).—Ribonucleic acids as indicated by the dark blue color with toluidine blue after conditioning with orange G were found to be present in large quantity throughout the cycle of the parasite. No spores which could be construed as dormant were found. The distribution of nucleic acids in the spores was markedly different, however, at the different developmental stages of the fungi. In normal sporangiospores (2 to 3 μ diameter) these substances were concentrated in a spherical or crescent-shaped central body measuring a fraction of a micron. The thick "capsule" of sporangiospores did not stain. In spherules of somewhat larger size (3 to 8 μ diameter) the toluidine blue reaction appeared at the periphery of the cell, and was seen as a thin, beaded boundary. In spherules slightly older than these, an outer wall which did not stain could be discerned by reduced illumination. All maturing spherules and sporangia had such walls, separating the Korson-positive material from the tis-

sues of the mouse. The cytoplasm of large precleavage spherules was often evenly and uniformly Korson-positive. Only the vacuoles and walls of such forms contained no ribonucleic acids.

In sections stained after treatment with perchloric acid a good deal of the positive material had vanished. The results of the test for DNA were irregular or weakly positive in forms ranging in size between sporangiospores and cleavage spherules. But the central bodies of sporangiospores, presumably nuclei, although remaining the same in form as before perchloric acid, were now green in hue, indicating DNA (fig. 1). In some of the cleavage spherules the cytoplasm appeared to be multinucleate and coenocytic. In yet other spherules in the late stages of cleavage the multiplying endospores were seen to contain from one to four nuclei (fig. 2). It is of interest that after treatment with perchloric acid, a subcuticular blue-staining portion of the sporangial walls was observed.

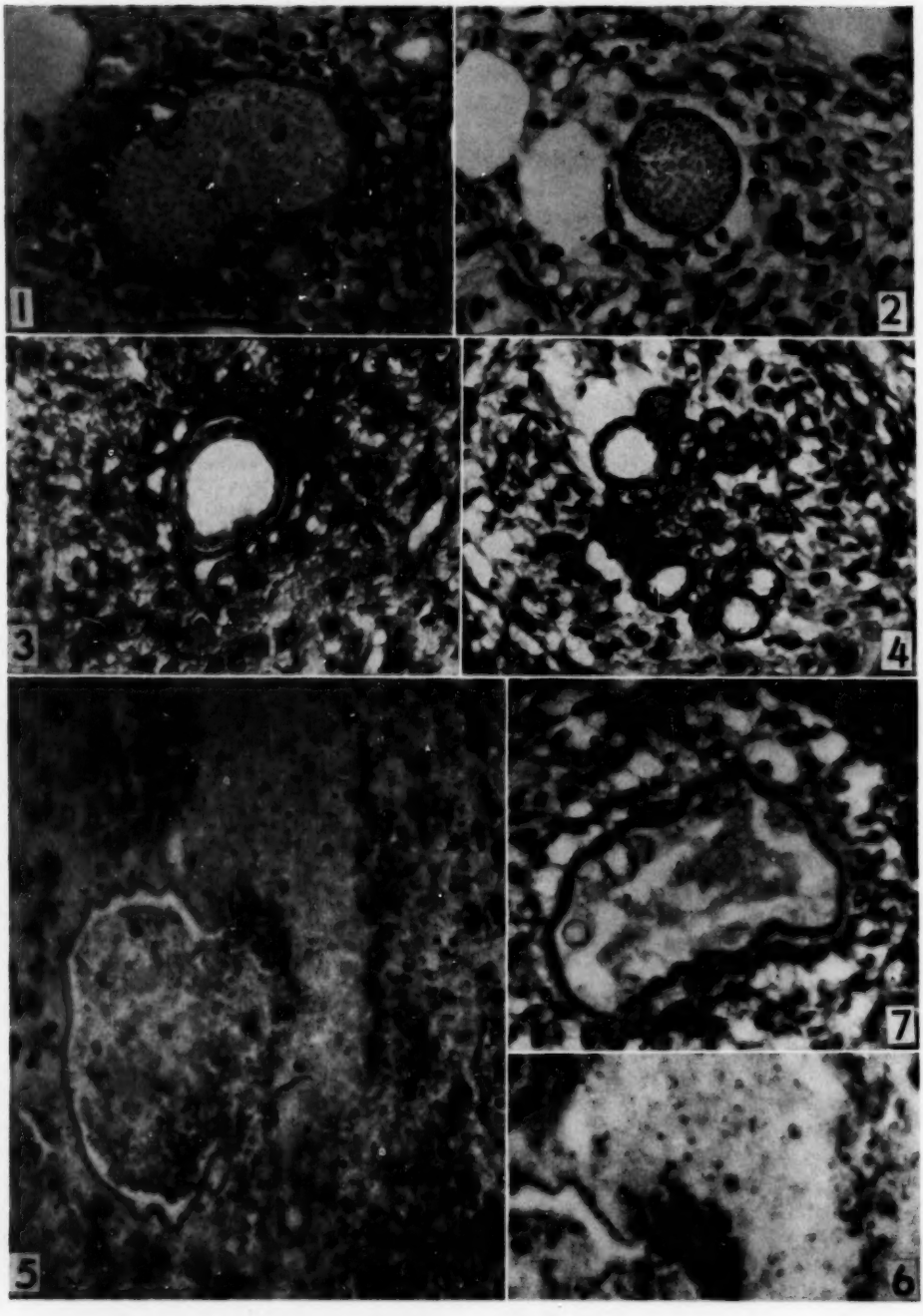
Occurrence of phospholipid (Baker stain).—The thick walls of all of the larger spherules were strongly positive for phospholipid (fig. 3), while the capsule or covering of sporangiospores was completely lacking in this substance. Other small spores, identical with those in which the nucleic acids were seen above to be distributed peripherally, had very thin outer layers containing phospholipid, the thinness of the wall being generally in proportion to the dimensions of the spore (fig. 4). Pretreatment with fat solvents removed the major portion of the positive material.

Mucopolysaccharide.—The location of mucopolysaccharide in the spherules was of particular interest. The Lison technique indicated that the capsule or covering of the sporangiospore, comprising practically the entirety of extranuclear structure, and the matrix material in which such spores are lodged until expelled from the sporangia both contained considerable mucopolysaccharide (fig. 5 and 6). Vacuoles within some of the larger spherules also contained Lison-positive material. But in these, and in all other types of spherules except sporangiospores, the mucopolysaccharide was separated from the tissue of the host by cytoplasm and the outer wall. Pretreatment of the sections with potassium hydroxide removed all pink-staining material.

Cellulose and chitin.—The walls of the large spherules, the shell of ascaris eggs, and the cellulose of privet leaf stained green with safranin-fast green. The cuticle of ascaris and the lignin of the plant material took the safranin. Digestion with Schweitzer's reagent destroyed the cellulose of the leaf although the lignin remained, but did not alter the staining or general appearance of sections of *C. immitis* or ascaris. The shell of the ascaris egg disappeared on treatment with sodium hypochlorite, while the staining of the privet leaf remained the same. The wall of the larger spherules of *C. immitis* was not dissolved by hypochlorite like the ascaris shell, but it lost its strong affinity for the fast green and stained pink and pale green in a mottled pattern. Of particular interest was the emergence after the hypochlorite treatment of a thin subcuticular layer with an affinity for safranin like that of the proteinaceous cuticle of ascaris or the lignin of the leaf (fig. 7).

DISCUSSION AND CONCLUSIONS

With Korson's technique, nucleic acids were seen to be plentiful in spherules at all stages of development, pointing to a state of continuous positive metabolism. The distribution of the positive-reacting material in the spher-


1
2
3
4
5
6
7

ules was markedly different in different spherule types, also indicating that the life processes of the fungus proceed at a rapid pace, with no resting stage of the cycle. As no cells which could be construed as dormant were found, the authors conclude that differences in histopathologic response to different classes of spherules cannot be explained on the basis that some of them exert their chief influence on the host as inert foreign bodies.

It is of interest that desoxyribonucleic acid appeared to be concentrated in the small central, spherical or crescent-shaped body of sporangiospores. Normal sporangiospores at the time of dehiscence of the sporangium contained one such "nucleus," while the cytoplasm of units within spherules which were still undergoing cleavage contained from one to several nuclei. The cytoplasm of a few large spherules, interpretable as precleavage forms of the fungus, appeared to be coencoytic and multinucleate. It was impossible to locate DNA with any certainty in spherules at stages between the sporangiospore and the spherule in cleavage. The entire spherule in such cases, with the exception of the wall, appeared to be evenly and weakly Korson-positive. In general, the distribution of the nucleic acids seen in this experiment agreed with the observations of Brachet[13] concerning nucleoproteins. During metaphase DNA localizes and increases at the expense of RNA, while the interphase states of the cell are characterized by predominance of RNA.

The Baker technique demonstrated the presence of considerable phospholipid in the walls of all spherules except sporangiospores. The thickness of the layer of phospholipid was seen to be proportional to the size of the cell. It is concluded on the basis of this observation that chemical interchange between spherules and host tissues is different in the case of sporangiospores where there is no interposition of a phospholipid layer, and that this may correlate with the polymorphonuclear reaction most prominently seen in the vicinity of sporangiospores in tissue sections containing *C. immitis*.

Menkin[6] has shown that the pH of an area of inflammation may condition the dominant form of leukocytes. He found that polymorphonuclear cells cannot exist in acid medium. The hydrogen ion reaction in lesions where sporangiospores are released may shift, owing to substances otherwise retained within the sporangial wall. The phospholipid of the wall itself may, on the other hand, possess distinctive chemotactic properties for the mononuclear elements of inflammation.

The extranuclear portions of the sporangiospores of *C. immitis* and the matrix material of sporangia contained

←

FIG. 1.—Sporangiospores emerging from sporangium of *C. immitis*. (Note uninucleate condition of majority of spores.) Korson stain. ×600.

FIG. 2.—Binucleate sporangiospores within an immature sporangium. Korson stain after perchloric acid treatment. ×600.

FIG. 3.—Phospholipid in wall of large spherule. Baker stain (extranuclear dark blue color locates phospholipid). ×600.

FIG. 4.—The thickness of the phospholipid layer is proportional to the dimensions of the spherules. Baker stain. ×600.

FIG. 5.—The "capsules" of sporangiospores and the matrix material of the sporangium contained mucopolysaccharide (pink color). Lison stain. ×600.

FIG. 6.—Pink-staining "capsules" of sporangiospores. Detail from figure 5. Lison stain. ×900.

FIG. 7.—Basophilic subcuticular layer of spherule wall (see also fig. 2). Safranin-fast green stain after chlorine treatment of section. ×600.

considerable quantities of mucopolysaccharide, according to the Lison indicator. In no other types of spherules was mucopolysaccharide a consistent feature, and when present at all in other types of spherules it lay in vacuoles insulated from direct contact with the tissues of the host by cytoplasm and an outer wall. The rapid change in the histopathologic reaction of the host, marked by the influx of polymorphonuclear leukocytes when sporangiospores are released, may be accounted for if the reddish staining matter of sporangiospores and matrix indicates the presence of a catalyst such as hyaluronidase or leukotaxine (permeability factor), both of which appear to possess prosthetic groups of a carbohydrate nature.[6,16]

The composition of the covering or wall of *C. immitis* has been the subject of considerable investigation. Utilizing X-ray analysis, Frey[24] has recently found that the Phycomycetes, with which *C. immitis* has been classified, fall sharply into two groups, those whose walls are chitin, and those whose walls contain a substance whose X-ray pattern agrees with that of cellulose. Frey was unable to find a juxtaposition of chitin and cellulose in any of the Phycomycetes investigated. As important phylogenetic significance has been attached to Frey's observations it seemed desirable to attempt to ascertain whether *C. immitis* should be placed in the chitin or the cellulose group of Phycomycetes. Along with sections of *C. immitis*, controls of known chitin and cellulose were subjected to treatment with Schweitzer's reagent and with chlorine. The results indicated the absence of cellulose in the walls of *C. immitis* when the treated slides were stained with safranin-fast green. Chlorine did not completely destroy the fungus wall, as it did the shell of ascaris (chitin control). But the affinity of the fungus wall for fast green was markedly altered by the chlorine, which unmasked a subcuticular layer of red-staining matter. This observation has suggested that chitin had been present, and largely removed by the chlorine from a basophilic matrix of protein nature.*

24. Frey, R. 1950, Chitin and cellulose in the cell walls of fungi. Ber. schweiz. botan. Ges. **60**: 199–230.

SUMMARY

1. No stage of dormancy in the parasitic cycle of *Coccidioides immitis* was demonstrable by use of the Korson stain for nucleic acids. All of the spherules appeared to be in an active state of metabolism. There are evidently no living spores of *C. immitis* which exert their influence on the host in the sole capacity of foreign bodies.

2. The presence of considerable phospholipid in the walls of large spherules, according to the Baker stain, and its absence in the small fungus cells suggest that differing permeabilities in the two spherule types may account for the differential histopathologic reaction to them.

3. The α-toluidine blue reaction of Lison indicated that sporangiospores differ from all other spherules of *C. immitis* in the possession of a relatively thick outer layer rich in mucopolysaccharide.

4. Treatment with Schweitzer's reagent did not alter the appearance of the wall of *C. immitis* when stained with safranin-fast green, while treatment with sodium hypochlorite destroyed known chitin (ascaris egg shell) and markedly changed the staining affinities of the fungus wall, revealing a basophilic matrix.

* It appears unlikely that this matrix might contain lignin. The authors recently performed controlled chlorine-sodium sulfite tests for lignin[22] on sectioned spherules with negative results.

THE EFFECT OF MAPHARSEN ON THE GLUCOSE METABOLISM OF *TRYPANOSOMA EQUIPERDUM*

WILLIAM CANTRELL

From the Department of Pharmacology, The University of Louisville School of Medicine, Louisville 2, Kentucky

The high rates of glucose consumption observed in trypanosomes, together with theoretical considerations based on the role of sulfhydryl-containing enzymes in glycolysis and the ability of arsenical drugs to combine with sulfhydryl groups, have led some workers to suspect that arsenical trypanocides act by disrupting the glycolytic process.

Chen[1] found that mapharsen, meta-amino-para-hydroxy-phenyl-arsenoxide, at a concentration of 1 mg per ml, inhibited hexokinase activity of lysed *Trypanosoma equiperdum* preparations 21% and 2 mg per ml inhibited it 91%. He found that mapharsen at 2.4 mg per ml inhibited the 3-phosphoglyceraldehyde dehydrogenase system 58% and 55% in two experiments. He also found that 0.2 mg. per ml inhibited adenosine-triphosphatase activity of lysed trypanosomes.

Marshall[2] found that the similar arsenical compound phenyl-arsenoxide strongly inhibited the utilization of glucose by whole blood containing *Trypanosoma evansi* when the drug was added at a concentration of $6\times10^{-4}M$. He concluded that this drug inhibited the hexokinase reaction in *T. evansi*, blocking the utilization of glucose at the first step, conversion of glucose to glucose-6-phosphate.

The present report describes experiments made to determine whether trypanosomes from mapharsen-treated rats use less glucose or produce less pyruvic acid than untreated trypanosomes.

METHODS

Glucose was determined by the method of Folin and Malmros,[3] in which the glucose is oxidized with alkaline potassium ferricyanide and the ferrocyanide produced is converted to Prussian blue, which is measured photometrically. Hawk, Oser and Summerson[3] suggest that the reagent blanks be ignored in using the method of Folin and Malmros because in the presence of a blood filtrate the blank density is reduced. That this procedure has something to recommend it can be demonstrated by simultaneously determining glucose in blood by the method of Folin and Malmros and by Nelson's[4] modification of Somogyi's method, which is said to be more specific for glucose. In such comparisons, if blanks are subtracted in the former method, the results are usually lower than results obtained with Nelson's method.

On the other hand, we have found that the reagents do contribute, to known glucose solutions, a reading equal to the reading obtained with the reagents in the absence of glucose. Therefore, changes in glucose concentration are better measured by subtracting the blank reading from the glucose standard reading. The change in glucose concentration can then be obtained from the change in colorimeter reading divided by the glucose standard reading minus the blank. Or, alternatively, the same result is obtained by subtracting the blank from all the readings and getting the "apparent" glucose concentrations at the beginning and end of the test period. Although these apparent values are

Received for publication October 2, 1952.

This work was aided by a grant to the University of Louisville by the Kentucky State Medical Research Commission.

1. Chen, G. 1948, J. Infect. Dis. **82**: 226–230.
2. Marshall, P. B. 1948, Brit. J. Pharm. & Chemotherap. **3**: 8–14.
3. Hawk, P. B., Oser, B. L. and Summerson, W. H. 1947, Practical Physiological Chemistry, ed. 12, Philadelphia, The Blakiston Co., p. 526.
4. Nelson, N. 1944, J. Biol. Chem. **153**: 375–380.

TABLE 1.—*Changes in glucose concentration in blood containing 53,000 to 134,000 trypanosomes per cubic mm and in uninfected rat blood incubated at 38 C*

Treated					Untreated				
Trypanosomes per cubic mm in thousands	0 min.	15 min.	25 min.	30 min.	Trypanosomes per cubic mm in thousands	0 min.	15 min.	25 min.	30 min.
Infected rat blood									
113	101*	38		9	94	118	65		9
77	106	51		7	134	101	18		11
88	100	45		10	77	107	51		11
93	114	50		9	79	105	58		20
72	117	64	33		72	129	86	62	
107	134	65	28		74	114	61	31	
56	120	74	49		53	130	80	50	
70	109	49	24		67	106	43	10	
63	108	56	24		58	117	56	25	
70	111	58	46		59	109	68	42	
Uninfected rat blood									
	128	108		91		143	121		105
	117	97	92			104	82		68

* Mg of glucose per 100 ml of blood.

not the closest possible estimates of the true values obtainable from the data, the difference between them is the closest estimate of the change in glucose concentration obtainable from the data. Therefore, we have used such values in table 1 to show the changes in glucose concentration. Pyruvic acid was determined by the method of Lu,[5] as modified by Bueding and Wortis[6] and Elgart and Nelson[7] and described by Burris, Umbreit and Stauffer,[8] in which pyruvic acid is converted to its 2,4-dinitro-phenylhydrazone, which is measured photometrically. Trypanosomes were counted in a hemocytometer in blood diluted 1:100 with physiological saline.

EXPERIMENTAL RESULTS

A rat with 108,000 trypanosomes per cubic mm of blood was treated with 2 micromoles of mapharsen per kg (0.516 mg per kg). Table 2 shows the course of the parasitemia during the next 2 hours.

The time required to clear half the trypanosomes from the blood was about 75 minutes, and the time required to clear 90% was about 108 minutes. During the first 45 minutes the number of trypanosomes was reduced about 10%.

With this sequence in mind, experiments were made to determine whether trypanosomes from mapharsen-treated rats use less glucose in the period just before they begin to disappear from the blood in large numbers. In each of these experiments two rats, infected at the same time and having about the same degree of infection, were used. One of each pair was treated intravenously with two micromoles of mapharsen per kg and the other served as a control. Blood was obtained 30 minutes after treatment and was defibrinated by shaking with glass beads. A sample was removed for counting of trypanosomes and glucose determinations. The rest of the parasitized blood was shaken in Warburg flasks in a 38 C water bath. Samples were removed at

TABLE 2.—*The effect of 2 micromoles per kg of mapharsen on the number of* T. equiperdum *in the blood.*

Time after injection	Trypanosomes per cubic mm
15 min. before	110,000
0	108,000
15 min. after	116,000
31	104,000
56	97,000
62	76,000
90	37,000
110	8,000

5. Lu, G. D. 1939, Biochem. J. **33**: 249–254.
6. Bueding, E. and Wortis, H. 1940, J. Biol. Chem. **133**: 585–591.
7. Elgart, S. and Nelson, N. 1941, J. Biol. Chem. **138**: 443–444.
8. Umbreit, W. W., Burris, R. H. and Stauffer, J. F. 1947, Manometric Techniques and Related Methods for the Study of Tissue Metabolism, Minneapolis, Burgess Publishing Co., p. 105.

TABLE 3.—*Glucose consumption by trypanosomes from mapharsen-treated rats and from untreated rats.*

Experiment	Treated	Untreated
1	38 mg	35 mg
2	45	47
3	40	47
4	47	34
5	47	32
6	46	45
7	46	57
8	44	64
9	51	71
10	47	36
Mean ± standard error	45.1 ± 1.2	46.8 ± 4.1

These figures in terms of glucose used per 10^{10} trypanosomes in 15 minutes.

15 minutes and at 25 or 30 minutes for analysis. The latter samples served to show that glucose continues to disappear at a fairly constant rate for at least 25 minutes under the conditions of the experiment. Blood containing about 50,000 to 100,000 trypanosomes per cubic mm was used to get appropriate rates of glycolysis. Since uninfected rat blood is glycolytic, it was necessary to allow for the action of the blood in calculating the amount of glucose used by the trypanosomes. It was found that blood from young rats (100 to 200 g) used glucose at the rate of about 20 mg per 100 ml in 15 minutes whether from treated or untreated rats. Therefore, this amount was subtracted from the observed changes in glucose concentration of infected blood.*

Ten pairs of infected rats were studied. Each pair consisted of a treated and an untreated rat. Table 1 shows the changes in blood glucose observed. The results of these experiments are shown in table 3 in terms of the amount of glucose used by the trypanosomes in 100 ml of blood containing 100,000 trypanosomes per cubic mm in 15 minutes. The figures therefore represent the glucose consumption of 10^{10} trypanosomes in 15 minutes. The results for treated and untreated trypanosomes are quite similar. About 45 mg of glucose was used per 10^{10} trypanosomes in either case. Chen and Geiling[9] found that 10^{10} trypanosomes used 52 mg of glucose in 15 minutes in Ringer's solution plus plasma.

These experiments furnished evidence that mapharsen does not exert its primary lethal action on the enzymatic process that is involved in the disappearance of glucose. They did not give a complete answer to the question of whether the drug affects glucose metabolism, because some step in the breakdown of glucose to pyruvate might be involved without affecting the removal of glucose itself. To check this possibility another series of experiments was made in which the amount of pyruvate produced was measured in infected blood from treated and untreated rats. Again it was necessary to study the behavior of uninfected blood. The amount of pyruvate appearing in rat

* We have recently measured glycolysis in uninfected rat blood from a series of five mapharsen-treated and five untreated rats without ever observing rates as high as those found in the two pairs done at the time of the trypanosome experiments. The mean values in these more recent experiments were 10.4 mg per 15 minutes for the untreated and 10.6 mg per 15 minutes for the treated bloods.

Another way of estimating the amount of glycolysis to be attributed to the blood is to plot the number of trypanosomes in the blood against the amount of glucose disappearing. When this is done the regression lines intercepted the zero ordinate near 20 mg percent, not 10 mg percent. Recently another experiment with infected blood was made, taking great care to obtain a good estimate of the number of trypanosomes. The value for glucose disappearing fell just 10 mg percent below the regression line previously drawn.

All these considerations led us to suspect that some new factor influencing glycolysis by blood was introduced between the two series of observations and that 20 mg percent is the better estimate of the glycolysis attributable to blood in the experiments with trypanosomes.

9. Chen, G. and Geiling, E. M. K. 1945, J. Infect. Dis. **77**: 139–143.
10. Reiner, L. and Smythe, C. V. 1934, Proc. Soc. Exper. Biol. & Med. **31**: 1086–1088.

blood incubated for 15 minutes was quite variable but was always small in comparison with the amount produced in parasitized blood. The mean change in unparasitized blood was less than 1% of the changes measured in parasitized blood, so the action of the blood does not affect the results very much. Both treated and untreated unparasitized bloods were studied. The amount subtracted from the observed changes was 0.23 mg per 100 ml. The results are given in table 4. If the experiments are considered in pairs as they were done, in 9 of 10 pairs the treated trypanosomes produced less pyruvate than the untreated ones. The difference was such that the untreated trypanosomes produced 1.7 moles of pyruvic acid from each mole of glucose, while the treated trypanosomes produced 1.4 moles of pyruvic acid from each mole of glucose.

These results agree reasonably well with those of Reiner and Smythe[10] with regard to the amount of pyruvate produced per mole of glucose although their experiments were made with trypanosomes in Ringer's solution while ours were in defibrinated blood. They found that 1.8 moles of pyruvate were produced for each mole of glucose used in one series of experiments and 1.74 moles in another series.

TABLE 4.—*Pyruvic acid production by trypanosomes from mapharsen-treated rats and from untreated rats*

Experiment	Treated	Untreated
1	20 mg	31 mg
2	24	29
3	28	48
4	41	36
5	33	41
6	29	40
7	36	44
8	33	33
9	33	36
10	32	42
Mean ± standard error	30.9 ± 1.9	38.0 ± 1.9

These figures are in terms of pyruvic acid produced per 10^{10} trypanosomes in 15 minutes.

DISCUSSION

It is interesting to compare the concentrations of drug used by Marshall and Chen in their in vitro experiments with the maximum concentration of mapharsen that can occur in our experiments. The intravenous injection of 2 micromoles per kg could give a maximum concentration of around 20 micromoles per liter of blood or $2\times10^{-5}M$ or about one-thirtieth as much as in the experiments of Marshall. Furthermore, $2\times10^{-5}M$ is equivalent to 0.00516 mg per ml. Since the concentrations used in Chen's experiments were usually 1 to 2 mg per ml., the drug concentrations in our experiments seem to be of a different order of magnitude. It is well known, however, that trypanosomes concentrate arsenic and, for this reason, perhaps the concentration of drug at its site of action is not as different as this comparison would indicate. The trypanosomes would need to raise the concentration of mapharsen at its site of action to a level 500 to 1000 times that of the surrounding medium to bring it into the range of Chen's experiments.

Since the trypanosomes in our experiments were taken from the rat at a time just before the death of large numbers of the trypanosomes could be expected to occur, we think these results convincingly rule out an action on hexokinase as the lethal action of mapharsen. Furthermore, we wonder whether the lethal action of the drug should be attributed to its effect on subsequent steps in the breakdown of glucose to pyruvate because 82% of the normal amount of pyruvate still was produced by the treated trypanosomes. The possibility that the drug exerts its primary lethal effect on some other phase of metabolism should be considered.

STUDIES ON THE PATHOGENESIS AND IMMUNITY OF TULAREMIA

I. THE DEMONSTRATION OF A PROTECTIVE ANTIBODY IN MOUSE SERUM

LOLITA PANNELL* AND CORA M. DOWNS

From the Department of Bacteriology, University of Kansas, Lawrence, Kansas

In tularemia, as in other infectious diseases, the antibody content of the circulating blood is not a reliable index to the resistance of the host. Killed culture vaccines induce the formation of agglutinins and precipitins against *Bacterium tularense* antigens in rats, mice, guinea pigs, and rabbits, but only in the case of the rat is the animal thereby protected against challenge with fully virulent organisms. Attempts in this laboratory to correlate protection against challenge with bactericidal tests on shed blood have been largely negative. Foshay[1] and Alexander[2] have correlated protective antibody with the precipitating capacity of antiserum, using carbohydrate as antigen. Larson[3] and Foshay[4] have shown that rats could be protected by the injection of antiserum, and Foshay[5] states that immune goat serum has therapeutic value in man. Francis and Felton[6] however, were unable to show protection in mice after injection with antiserums from rabbit, horse, sheep, and man. All attempts in our laboratory to develop a protective test for mice using virulent challenge strains have been negative. Since mice are protected from multiple lethal challenge doses after they have recovered from infections with *Bact. tularense* of low virulence,[7] and since rats are more solidly immune after recovery from infection than after vaccination with killed cultures,[8] it seemed desirable to try to develop a reliable test which could measure the protective effect of a serum containing antibodies resulting from the injection of killed cultures or from infection, so that the effect of circulating antibodies could be correlated with the resistance of the intact animal to challenge.

We therefore proposed to try to develop a protective test in the white mouse, using serum from various sources and using a challenge strain of lowered virulence rather than a completely virulent strain.

Received for publication October 9, 1952.

This work was supported in part by a contract between the Office of Naval Research, Department of the Navy, and the University of Kansas, and by the United States Public Health Service.

* From a dissertation presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Kansas, 1950.

1. Downs, C. M., Coriell, L., Eigelsbach, H. T., Plitt, K. F., Pinchot, G. B. and Owen, B. J. 1947, Studies on tularemia. II. Immunization of white rats. J. Immunol. **56:** 229–243.
2. Downs, C. M. 1932, Immunologic studies on tularemia in rabbits. J. Infect. Dis. **51:** 315–323.
3. Ruchman, I. and Foshay, L. 1949, Immune response in mice after vaccination with *Bacterium tularense*. J. Immunol. **61:** 229–234.
4. Coriell, L. L., Downs, C. M. and Clapp, M. P. 1947, Studies on tularemia. III. Immunization of mice. J. Immunol. **56:** 245–253.
5. Foshay, L., Ruchman, I. and Nicholes, P. S. 1947, Anti-tularense serum: correlation between protective capacity for white rats and precipitable antibody content. J. Clin. Investigation, **26:** 756–760.
6. Alexander, M. M. 1950, A quantitative antibody response of man to infection or vaccination with *Pasteurella tularensis*, J. Exper. Med. **92:** 51–57.
7. Francis, E. and Felton, L. D. 1942, Antitularemic serum. Pub. Health Rep. **57:** 44–55.
8. Larson, C. L. 1947, A serum protection test in tularemic infections in the white rat. Pub. Health Rep. **62:** 1793–1800.

METHODS AND MATERIALS

Experimental animals.—The white mouse was selected as the test animal because its susceptibility to tularemia can be determined quantitatively and because it can be immunized by infection with strains of lowered virulence. The mice employed in these investigations were obtained from the Maple Grove Rabbitry, Springfield, Missouri. They were selected without respect to sex, and their weights approximated 20 g.

Strain of Bact. tularense.—The *Jap* strain was employed as the antigen for the production of immune mouse serum and as the challenge strain unless indicated otherwise. This strain was chosen because it represents a strain of moderate virulence, and because it is highly imunogenic in the white mouse in sublethal doses. It has an approximate LD_{50} of 500,000 organisms for the white mouse when inoculated intraperitoneally or subcutaneously. The *Jap* strain was isolated in Japan in 1926 from a human lymph node.

Mouse protection test.—The immune serum to be tested for protective capacity was obtained in the following manner: A saline suspension of the *Jap* strain of *Bact. tularense* was prepared from a 24-hour culture grown on glucose cysteine blood agar (GCBA) slants to give a 24% light transmission in the Coleman spectrophotometer at a wave length of 600. This standard suspension contained approximately 1 to 4×10^9 organisms per ml. Decimal dilutions were prepared in 0.85% sodium chloride solution. An immunizing dose of 0.5 ml of a 10^{-6} saline suspension containing approximately 50,000 organisms was inoculated intraperitoneally into each of the desired number of white mice. Each immunized mouse in this group was bled from the heart on either the 5th, 6th, or 7th post-inoculation day. The blood was pooled, kept at 4 C overnight, and the serum was separated on the following day. To demonstrate the protective capacity of the serum, equal quantities of this serum and the challenge dose of the *Jap* strain were mixed and inoculated immediately into white mice. The challenge consisted of 0.5 ml of a suspension of *Bact. tularense*, containing approximately 1×10^7 to 1×10^8 cells. The test mice were observed for a period of 10 days. The day of death was recorded and the mortality percentage was computed. Surviving mice were challenged with 1000 LD_{50} of the highly virulent *Schu* strain of *Bact. tularense*.

A mouse protection index was calculated in a manner similar to that of Meyer and Foster.[9] Meyer, in his work on the plague bacillus, designated the percentage mortality divided by the average time of death as the mouse protection index (MPI). A small number represents greater protection than a large number. Because of variation in susceptibility of the white mouse and variation in the time in which different strains of *Bact. tularense* killed white mice, all experiments were controlled by employing normal mouse serum. In this manner, it was possible to calculate an MPI for the immune serum employed, and an MPI for the normal serum employed. For further verification of the protective capacity of serum, a protective ratio (PR) was calculated. This is a direct comparison of the MPI of the normal serum to the MPI of the immune serum. A PR of 1 represents no protection.

Agglutination tests.—A formalinized suspension of the *38* strain of *Bact. tularense* was used as antigen. The turbidity of the suspension was prepared to match a standard suspension as defined above. Twofold saline dilutions of serum were made employing 0.2 ml transfers. The suspensions were kept at 4 C overnight and read on the following day.

Adsorption tests.—Immune serum was adsorbed by using a very heavy suspension of cells, living or killed as stated in the experiments, in a small volume of saline. The serum-cell suspension was kept overnight at 4 C. The serum was cleared of cells by centrifugation and a rapid slide agglutination test was performed to check for complete adsorption. When viable cells were employed, a sample of serum was plated to determine sterility of the supernate.

Serums tested.—In the routine mouse protection test the serum employed was obtained from immunized mice as stated above. The data pertaining to the serums used to test for cross-species protection were as follows: The rat serum was collected 6 weeks after immunization and showed an agglutinin titer of 1:80. The rabbit serum which had been stored for 14 months showed an agglutinin titer of 1:2560. The guinea pig serum was collected 8 weeks after immunization and showed an agglutinin titer of 1:2560. The human serum was collected 4 months after a subclinical infection and showed an agglutinin titer of 1:2560.

EXPERIMENTAL RESULTS

The following experiments were conducted in a study of the protective capacity of immune serum:

9. Meyer, K. F. and Foster, L. E. 1948, Measurement of protective serum antibodies in human volunteers inoculated with plague prophylactics. Stanford Med. Bull. **6**: 75–79.

TABLE 1.—*Development and duration of protective antibodies in mouse serum following active immunization.*

Inoculum: *Jap* serum and living *Jap* cells	Day	Test mice*			Control mice†			PR
		Dead / Tested	ADD‡	MPI	Dead / Tested	ADD‡	MPI	
1×10^7	1	6/6	4.5	22	6/6	4.1	24	1
1×10^7	2	6/6	4.1	24	6/6	3.6	28	1
1×10^7	3	4/6	5.8	11	6/6	4.6	22	2
1×10^7	5	2/6	5.0	7	6/6	3.1	32	5
1×10^7	6	3/6	5.3	9	6/6	5.0	20	2
1×10^7	7	3/6	7.3	7	6/6	3.6	28	4
1×10^7	8	2/6	10.0	3	6/6	3.8	26	8
1×10^7	10	3/6	8.0	6	6/6	5.6	19	3
4×10^8	21	9/10	4.1	22	3/3	1.0	100	5
3×10^8	24	5/8	5.0	13	3/3	2.5	40	3
4×10^8	28	10/10	5.8	17	5/5	1.6	63	4
3×10^8	35	8/8	4.7	21	4/4	2.0	50	2
3×10^8	42	3/6	8.0	6	3/3	1.6	63	10
2×10^8	49	3/3	5.3	19	4/4	3.7	27	1

* The test mice received *Jap* immune serum plus the inoculum indicated.
† The control mice received normal serum plus the inoculum indicated.
‡ Average day of death.

Development and duration of protection.—Each mouse of a group of normal mice was inoculated with 40,000 living organisms of the *Jap* strain. Ten to fourteen of these mice were bled on the day indicated in table 1. A mixture of 0.5 ml of serum and 0.5 ml of *Jap* organisms was inoculated intraperitoneally into 6 to 10 white mice. The results presented in table 1 indicate that there is apparent on the 3rd to 5th days some evidence of a protective capacity in immune serum which remained for at least 7 weeks. An irregularity in the degree of protection obtained may be noted.

Minimal amount of serum necessary for protection.—A series of protection tests was carried out on various amounts of serum ranging from 1.5 ml to 0.001 ml. The results are presented in figure 1. Serum in amounts of 0.01 ml protected mice against the challenge dose. The PR obtained with 0.0075 ml of serum was low, and the PR continued to decrease with a decrease in the amount of serum employed. No protection was obtained with 0.001 ml of serum. It is also seen from figure 1 that the degree of protection was not greatly augmented by an increase in the amount of serum employed, once a sufficient amount was introduced into the mouse to afford protection.

Comparison between protection and agglutinin content of immune serum.—An inoculum consisting of 43,500 living *Jap* cells was given to each of 200 white mice. Ten mice from this group were sacrificed daily, beginning on the 2nd day following the inoculation. Of these 10 mice, 3 were bled separately to determine the agglutinin titer. The excess serums from these 3 mice were added to the pool of serum obtained from the 7 mice. Protection tests were performed daily on this serum by the inoculation of 6 normal mice with a combination of 0.5 ml of a *Jap* suspension of approximately 1×10^8 cells and 0.5 ml of the immune serum. On the 15th postinoculation day, the remaining mice from the original group of 200 were given a "booster dose" of approximately 4×10^6 *Jap* cells. Agglutination tests and protection tests were performed daily on the serums of these mice for the following 7 days. The results of this experiment are presented in figure 2.

From these results it is apparent that protection is evident before an agglutination titer is demonstrable. The protective substance is present on the 3rd day and rises sharply on the 4th day.

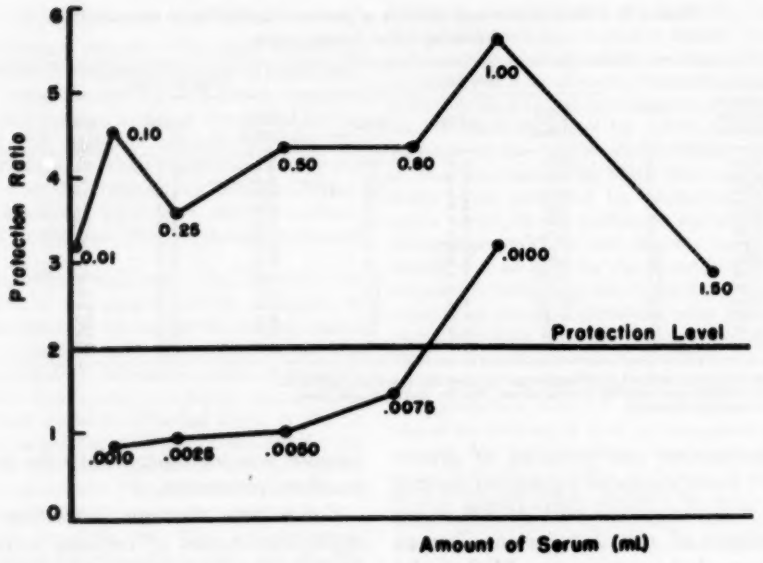


FIG. 1.—Minimal amount of serum necessary for protection. Line indicates the protective ratio obtained with amount of immune serum employed, as indicated at the respective points.

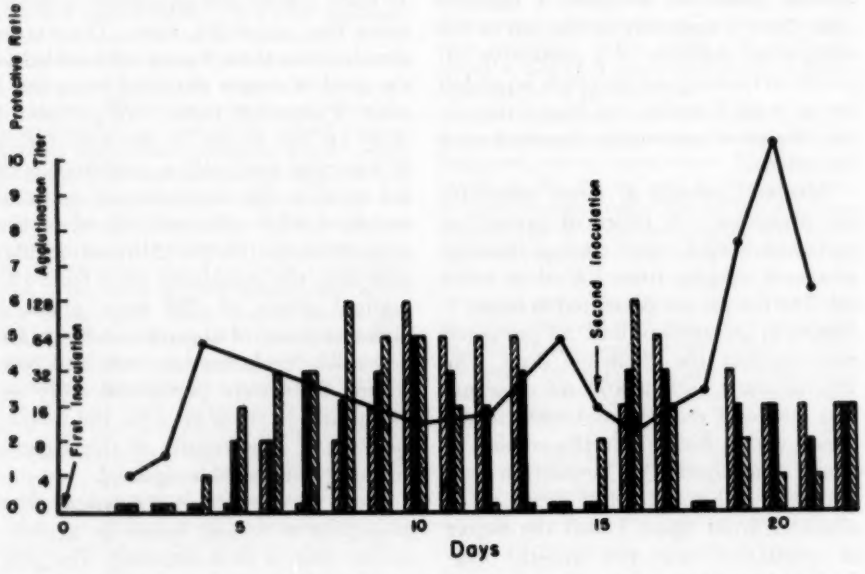


FIG. 2.—Comparison of agglutinins and protective antibody. Line indicates the protective ratio. The bars indicate the average agglutinin titers of 3 mice.

The first evidence of an agglutinin titer is seen in 1 out of 3 mice on the 4th day, 2 out of 3 mice on the 5th day, and 3 out of 3 mice on the 6th day. The agglutinin titer of the serums of the 3 mice tested reached a peak on the 10th to the 13th day. Following the booster dose, the response in titer was immediate, with a peak reached on the day following re-inoculation. The protective capacity remained in evidence throughout the daily tests. In response to the booster dose, the protective capacity increased gradually to reach a peak on the 5th day following re-inoculation. Neither the agglutinin titer nor the PR reached a negative point at the termination of the experiment. The variation found in agglutinin response by the individual mouse is apparent.

Heat stability of the protective substance.—Immune serum was collected from mice which had survived an inoculation of the *Jap* strain. This serum was divided into 4 equal parts. One pool of serum remained untreated. The other 3 were heated for one-half hour at 56, 60 and 65 C, respectively. The 4 pools of serums were then added in equal portions to saline suspensions containing 3.4×10^8 *Jap* cells. The results of the protection tests carried out on the heated and unheated serums showed that there was no significant loss in protection due to the temperatures used. The average MPI of the heated serums was 11, the PR 3, whereas the MPI of the controls was 36.

Cross-species protection.—Immune serums obtained from the rat, rabbit, guinea pig, and human, as described above, were employed in the routine protection test with the mouse. The PR's obtained, as seen in table 2, indicate excellent protective effect of all serums against infection in the mouse with *Bact. tularense.*

Adsorption of the protective substance.—The adsorption process was conducted as previously described. To test the protective capacity of adsorbed and unadsorbed immune serums, equal volumes of each were added to a 10^{-1} dilution of a standard suspension of *Jap* cells and used as inoculums for 2 groups of normal mice. Adsorbed and unadsorbed normal mouse serums were used as controls. The organisms used for adsorption were living *Jap* cells, boiled *Jap* cells, or living *38* cells. It is evident from the results shown in table 3 that the protective substance was adsorbed from immune serum either by viable or dead cells of the homologous strain or viable heterologous cells.

Killed cells for the production of protective serum.—Since it has been found[10] that an original inoculum of 10^4 *Jap* cells multiplies in the mouse to approximately 10^6 cells (combined number in heart blood, spleen, and liver) within a 5-day period, an inoculum of this order of boiled *Jap* cells was used to approximate the height attained by multiplication. A suspension of living *Jap* cells approximating 4×10^4 cells per ml was employed for control purposes. Two groups of 25 mice each were inoculated intraperitoneally with the 2 suspensions.

On the 5th postinoculation day, 5 mice from each group were challenged with 2000 LD_{50} of the *Schu* strain. There were no survivors in the group of 5 mice which had received the boiled *Jap* cells. There were no deaths in the group of 5 mice which had received the living *Jap* cells.

The remaining 20 mice in each group were bled from the heart on the 5th postinoculation day. The serums which were collected into 2 pools were added

10. Downs, C. M. and Woodward, J. M. 1949, Studies on pathogenesis and immunity in tularemia. III. Immunogenic properties for the white mouse of various strains of *Bacterium tularense.* J. Immunol. **63**: 147–163.

TABLE 2.—*Protective capacity of serum from various species for the mouse.*

Serum* and dose of *Jap* cells	Test mice			Control mice			PR
	Dead / Tested	ADD†	MPI	Dead / Tested	ADD†	MPI	
Rat +2.7×10^9	17/20	5.0	17	10/10	1.2	83	5
Rat +2.7×10^8	8/20	5.6	7	10/10	1.6	63	9
Rabbit +3×10^8	18/20	4.2	21	10/10	1.3	77	4
Rabbit +3×10^7	7/20	6.7	5	9/10	3.0	30	6
Guinea pig +4.4×10^9	13/20	5.3	12	5/5	1.0	100	8
Guinea pig +4.4×10^8	6/19	2.3	14	10/10	1.3	77	6
Human +1.5×10^9	11/20	5.3	10	6/6	2.0	50	5

* Agglutinin titers of the serums employed were: rat—1:80, rabbit—1:2560, guknea pig—1:2560, human—1:2560.
† Average day of death.

to equal volumes of a saline suspension containing 10^8 *Jap* cells for the usual mouse protection test. The results showed that a protective serum may be produced equally well by living or killed *Jap* cells as antigen, since the MPI of serum produced with boiled cells was 21, the PR 3, whereas the MPI of the serum produced with living cells was 14 and the PR 4. The respective mortalities were 85 and 75%. The MPI of the control serum was 56 and the mortality 100%.

Avirulent strain 38 *as antigen for the production of protective serum.*—It was of interest to compare a strain known to be incapable of producing a clinical infection in the white mouse with a strain of known infectivity by using each strain as antigen for a protective serum. Accordingly, 2 groups of mice were inoculated with 10^4 *Jap* cells and 10^8 *38* cells, respectively. The routine protection test was conducted on the serums obtained from the 2 groups. Good protection was obtained from either pool of serum. The MPI for *Jap* antiserum was 5 and the PR 15. The mortality was 29%. For the *38* antiserum, the MPI was 2, the PR 32, and the mortality 11%. Therefore, the *38* strain which is not sufficiently immunogenic in white mice to give active immunity against virulent strains appears to induce the formation of protective antibodies. The MPI of the normal serum was 77, with 100% mortality.

Minimal amount of antigen necessary to produce the protective serum.—Serial 10-fold saline dilutions of a standard suspension of *Jap* cells consisting of approximately 6 to 60,000 organisms were employed as antigen. Five days following the intraperitoneal inoculation of these amounts into 5 groups of mice, the serums were collected and used in

TABLE 3.—*Adsorption of the protective antibody from immune serum.*

Serum and challenge dose	Test mice*			Control mice†			PR
	Dead / Tested	ADD‡	MPI	Dead / Tested	ADD‡	MPI	
Unadsorbed immune serum +2.7×10^8 *Jap* cells	13/24	5.9	9	4/4	1.0	100	11
Serum adsorbed with living *Jap* cells +2.7×10^8 *Jap* cells	24/24	1.6	63	4/4	1.0	100	2
Serum adsorbed with boiled *Jap* cells +2.8×10^8 *Jap* cells	17/18	1.2	78	4/4	1.5	67	1
Serum adsorbed with living *38* cells +1×10^8 *Jap* cells	11/11	1.4	71	4/4	1.2	83	1

* Test mice received immune serum treated as indicated plus the inoculum shown.
† Control mice received normal serum treated as indicated for the immune serum plus the inoculum shown.
‡ Average day of death.

TABLE 4.—*Minimal effective inoculum of antigen for protective antibody response.*

Amount of antigen	Dead / Tested	ADD‡	MPI	PR
Test mice*				
6.7×10^4	3/7	5.3	8	7
6.7×10^3	2/4	5.5	9	6
6.7×10^2	4/7	6.0	10	6
6.7×10^1	9/9	1.3	77	1
6.7×10^1	9/9	1.4	71	1
Control mice†				
—	6/7	1.6	54	—

* Test mice received 0.5 ml of the "immune serum" obtained from mice inoculated with the amounts of *Jap* cells indicated plus 1×10^3 *Jap* cells.
† Control mice received 0.5 ml normal serum plus 1×10^3 *Jap* cells.
‡ Average day of death.

the mouse protection test. The data from table 4 indicate the minimal inoculum which produces a protective serum. A PR of 5.6 was obtained from serum produced by the injection of 6.7×10^2 cells. When 6.7×10^1 cells were used as the immunizing dose, the PR dropped to 0.69, indicating no protection. The data also indicate some relationship between increasing amounts of antigen and increasing PR.

Effect of alteration in the routes of inoculation on protection.—The usual manner of inoculation in the mouse protection test was the intraperitoneal inoculation of equal parts of serum and challenge doses simultaneously. In order to establish the fact that variation from this routine procedure had no effect on the protective capacity of immune serum, the following inoculations were made: Each mouse in the 1st group received 0.5 ml immune serum intraperitoneally into the lower left quadrant, followed immediately by 0.5 ml of the cell suspension, containing 10^8 *Jap* organisms, intraperitoneally into the lower right quadrant. Each mouse in the 2nd group received 0.5 ml of immune serum intraperitoneally into the lower left quadrant, followed immediately by 0.5 ml of the cell suspension subcutaneously over the lower left quadrant. Each mouse in the 3rd group was given 0.5 ml immune serum subcutaneously over the lower left quadrant, followed immediately by 0.5 ml of the cell suspension subcutaneously over the lower right quadrant. Each mouse in the 4th group received a mixture of serum and cells intraperitoneally into the lower left quadrant, employing the routine procedure. Each mouse in the groups of control mice received normal mouse serum and organisms by identical routes of inoculation. Since the MPI resulting from the various tests ranged from 7 to 13 and the PR from 10 to 5, it is apparent that the same degree of protection was conferred by the various modes of inoculation. The average mortality for the test mice was 46% and 100% for the control mice where normal serum was used. The MPI for the control mice ranged from 63 to 67.

DISCUSSION

The results reported indicate that mice receiving serum containing antibodies against *Bact. tularense* are protected against a challenge dose of living cells of lowered virulence, but this protection is very incomplete. There is variation in the degree of protection afforded by immune serum, and this probably is due to the content of a protective antibody in the immune serum which is not strictly correlated with the agglutinin content. It is necessary to use a pool of serum from large groups of immune mice in order to obtain a more stable trend.

The average MPI obtained for the test mice was 15.2 and for the control mice 57.6. Meyer[11] has stated from experiments on animals with *Pasteurella pestis* that the protective index should

11. Meyer, K. F., Foster, L. E., Baker, E. E., Sommer, H. and Larson, A. 1948, Experimental appraisal of antiplague vaccination with dead virulent and living avirulent plague bacilli. Proc. Fourth Internat. Cong. Trop. Med. & Mal. Washington, D. C., May 10–18, pp. 264–274.

be at least one-half of the normal index in order to obtain a high rate of survival from infections which are invariably fatal to controls. He also found[9] that an MPI below 10 in guinea pigs indicates a high percentage of survival in massive infections with the plague bacillus. In the case of *Bact. tularense* a challenge dose of the *Jap* strain usually killed 100% of the controls in 1 or 2 days. Immune serum did not protect 100% of the test mice against this dose, but the survival time and the survival percentage were increased noticeably from that of the control animals. An arbitrary limit was set for an acceptable numerical expression of protection. From the results obtained, it appeared that a PR above 2 indicated protection. More significance was attached to the ratio (PR) obtained by a comparison of the indices of control and test mice than the MPI of either alone.

In comparison of the protective antibody and agglutinins several differences were noted. The protective antibody appeared between the 3rd and 5th day, while demonstrable agglutinins did not appear until the 5th or 6th day following inoculation. The protective capacity lasted at least 7 weeks, but became more irregular toward the latter part of the test period. Demonstrable agglutinins, however, disappeared after 15 days following one injection of 4×10^4 *Jap* cells (fig. 2). The agglutinin response was immediate following a 2nd inoculation, but a rise in the protective antibody content was not apparent until 3 days later. The response of agglutinins, in general, was not as great following the booster dose as the initial response. These results suggest that the 2 antibodies are a separate response, and that the protection derived from immune serum is not correlated with the agglutinin titer. These findings are in agreement with the results obtained by other workers.[3,5,7] Ruchman and Foshay[3] have reported on an intensive study of the agglutinins and active immunity to challenge in mice after vaccination with *Bact. tularense*. Following a single dose of phenolized suspension of the *Schu* strain, they found that white mice developed a maximal agglutinin titer of 1:40 to 1:80 at 3 to 4 weeks. A booster dose administered 6 to 7 weeks after the initial vaccination resulted in a slight negative phase, followed by a peak of 1:640 to 1:1280 in 1 week. No resistance could be demonstrated in these mice against small numbers of living, virulent cells in spite of the accelerated immune response. The titers reported by these workers were considerably higher than those obtained in this study resulting from the injection of a sublethal dose of living cells. The immune response in the mice observed in this study appeared much earlier than the time reported by these workers and disappeared earlier. It has been shown that the immune state in the white mouse becomes evident 3 days after the immunizing infection with strains of lowered virulence.[10] This time coincides with the appearance of protective antibodies and suggests a close relation between active and passive immunity.

The results obtained from the experiments on species specificity showed that the mouse can be protected equally well by immune serum produced in the rat, rabbit, guinea pig, man or mouse (table 3). The antigen which is responsible for the production of this protective antibody appears, therefore, to show no host immunogenic specificity. Because a significant difference existed in the immunogenic activity of several strains of avirulent plague bacilli for the mouse and for the guinea pig, Meyer et al[11] were led to the discovery of the complex antigenic make-up of that organism.

A somewhat similar host immunogenic specificity is apparent in *Bact. tularense* in active immunity, in that strains which immunize rats do not immunize mice. This may indicate the possibility that antigenic differences between strains exist which are brought out by host differences, but which are not apparent in serological tests. However, it apears that the antigen responsible for the protective antibody in the strain employed is equally active immunogenically in the various species studied.

By the use of adsorption tests, it was found that viable or killed cells of the homologous or heterologous strain were able to adsorb the protective antibody from immune serum (table 3). These results indicate that a common antigen is involved in the production of protective serum. The fact that the avirulent *38* strain was able to adsorb the protective substance, although this strain cannot produce a clinical infection in the mouse and does not induce active immunity, indicates that the antibody may be only a small part of the complete immunity mechanism in this disease. The antigen which produces the protective substance is present in living or dead cells. The injection of heat-killed cells or phenolized cells can produce an antibody which will passively protect a normal mouse against the low and moderately virulent strains. However, the mouse which produced this protective antibody is not refractory to a challenge of a fully virulent strain. The injection of as little as 200 viable *Jap* cells as an initial dose enables the mouse to overcome 100 LD_{50} of the *Schu* strain.[10] Without this immunizing infection, the mouse is susceptible to 1 *Schu* cell. From previous work[10] it is known that all immunogenic strains of *Bact. tularense* multiply in the white mouse. It seems probable that in order to induce a state of active immunity against fully virulent strains, some process involved during multiplication of the organisms within the host must take place. The immunity conferred by passive transfer at best gives an incomplete immunity, expressed as partial protection against strains of relatively low virulence and little or no protection against fully virulent strains. It is known in the case of anthrax bacillus that the interaction of the growing organisms and the host fluids causes the production of a "protective" antigen.[12] This antigen gives rise to an active immunity which cannot be transferred passively. The evidence that solid immunity is conferred upon the host after infection with *Bact. tularense* points toward a similar mechanism but has not been demonstrated.

A striking similarity exists between the minimal effective antigen for the production of active immunity and the production of protective antibodies. It was shown[10] that 200 *Jap* cells as the initial dose served to confer a solid im munity to infection. From table 4 it is seen that 600 *Jap* cells were the minimal effective inoculum for the production of the protective substance. This number represents the threshold of stimulation below which no protection can be demonstrated. Since the work cited[10] indicated that stimulation of an immune response was possible with 20 *Jap* cells, it is apparent that the protection test is not sufficiently sensitive to detect the slight degree of immunity elicited by this small dose of cells. Although differences in antigenic structure of various

12. Watson, D. W., Cromartie, W. J., Bloom, W. L., Heckly, R. J., McGhee, W. J. and Weissman, N. 1947, Studies on infection with *Bacillus anthracis*. V. The isolation of an inflammatory factor from the crude extracts of lesions of *Bacillus anthracis* infection and its biological and chemical relationship to glutamyl polypeptide. J. Infect. Dis. **80**: 121–136.

strains of *Bact. tularense* have not been demonstrated by serological tests, work by Downs[13] and a report by Eigelsbach[14] have indicated that immunogenic and nonimmunogenic clones of *Bact. tularense* may be selected from various strains. It is possible, therefore, that the strains which have been reported as poor immunizing agents[10] are deficient in the immunogenic antigen or cannot multiply sufficiently in the animal to produce it. This has been proven to be the case with some avirulent strains of the plague bacillus.[11] In this case, antigen analysis of the different avirulent strains of poor immunogenicity in mice was shown to vary significantly in the content of fraction I, which is believed to be responsible for this activity.

Exactly what pathway the immunity mechanisms follow in a tularemic infection is not clear. It is apparent that union of the serum antibodies and bacterial cells before introduction into the animal is not necessary, since the evidence presented concerning the different routes of inoculation shows clearly that the route of inoculation of serum and organisms does not alter the protection afforded by immune serum.

Foshay has shown[6] that there is good correlation between the precipitin content of immune serum of rats and its protective capacity. This observation was made by employing the phenol-extracted polysaccharide of acetone-dried cells of *Bact. tularense*. More recently, Alexander[6] has furthered this observation with a study of the quantitative antibody response of infected or vaccinated humans. She was able to demonstrate that adsorption of immune serum with the phenol-extracted polysaccharide removed the protective capacity of the serum for rats. The fact that antiserum to *Bact. tularense* is protective for the rat or mouse can no longer be questioned. The protection test as reported in this paper with the use of a strain of lowered virulence for challenge and a uniformly susceptible host provides an instrument for observation of immunologic problems involved in this disease.

The results presented here indicate clearly that circulating antibodies provide a certain amount of protection to the susceptible animal, but that this degree of protection is insufficient to overcome infection with a virulent strain, whereas solid protection is conferred by recovery from infection with a strain of lowered virulence. Although circulating antibodies may play some role in protection, the most effective role appears to be played by the tissues of the intact animal.

13. Downs, C. M. and Moody, M. To be published.
14. Eigelsbach, H. T., Braun, W. and Herring, R. D. 1951, Studies on the variation of *Bacterium tularense*. J. Bact. **61**: 557–569.

SUMMARY

Protective antibodies against *Bacterium tularense* can be demonstrated by the injection of white mice with immune serums from mice, rabbits, rats, guinea pigs, and man, when a strain of lowered virulence is used for challenge. These protective antibodies are induced by killed or living cells of various strains of *Bact. tularense*, and can be removed by adsorption with homologous or heterologous strains. The degree of protection may be expressed by a mouse protection index or the protection ratio between normal and immune serum. The relation of these protective antibodies to agglutinins is discussed.

VOLUME 92 MARCH-APRIL 1953 NUMBER 2

The Journal of Infectious Diseases

TABLE OF CONTENTS

PAGE

JACK D. TIZARD. The migration, distribution in the brain, and growth of ascarid larvae in rodents 105

J. F. A. SPRENT. On the migratory behavior of the larvae of various ascaris species in white mice 114

LOUIS T. MILLER AND HARRY A. FELDMAN. Incidence of antibodies for toxoplasma among various animal species 118

NED B. WILLIAMS, ROBERT F. NORRIS AND PAUL GYÖRGY. Antigenic and cultural relationships of *Lactobacillus bifidus* and *Lactobacillus parabifidus* ... 121

ELEANOR JOHNSON TOBIE AND THEODOR VON BRAND. The development of arsenic resistance in *Trypanosoma gambiense* and its influence on parasitemia 132

L. W. SLANETZ AND CLARA H. BARTLEY. The diagnosis of staphylococcal mastitis, with special reference to the characteristics of mastitis staphylococci 139

WILLIAM BURROWS. Studies on immunity to Asiatic cholera. VI. Observations on the relation of antibody to effective immunity to experimental enteric cholera, with a note on sulfonamide therapy 152

WILLIAM BURROWS AND LAWRENCE L. WARE. Studies on immunity to Asiatic cholera. VII. Prophylactic immunity to experimental enteric cholera 164

J. E. UPTON, R. P. HANSON, D. DOW, AND C. A. BRANDLY. Studies on intracerebral inoculation of Newcastle disease virus (NDV) into mice. I. Response of weanling mice to 25 strains of NDV 175

J. E. TARBET AND A. M. BERNLAU. Histochemical investigation of the spherule of *Coccidioides immitis* in relation to host reaction 183

WILLIAM CANTERELL. The effect of mepharsen on the glucose metabolism of *Trypanosoma equiperdum* 191

LORETA PANNELL AND CORA M. DOWNS. Studies on the pathogenesis and immunity of tularemia. I. The demonstration of a protective antibody in mouse serum 195